



Enzymatic production of human milk oligosaccharides

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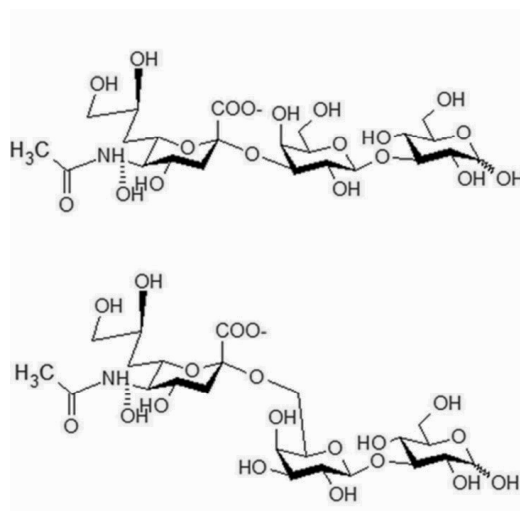
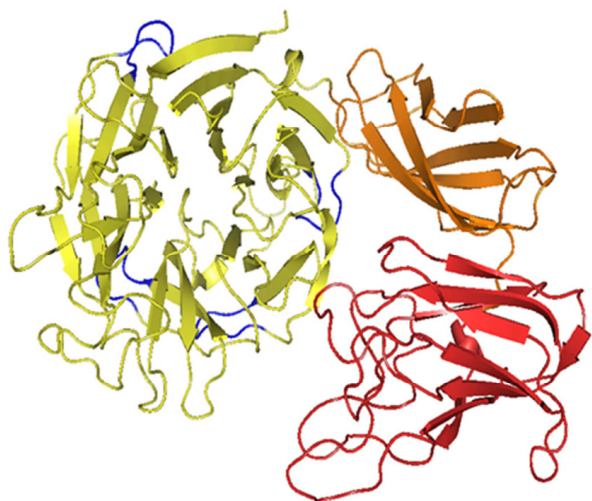
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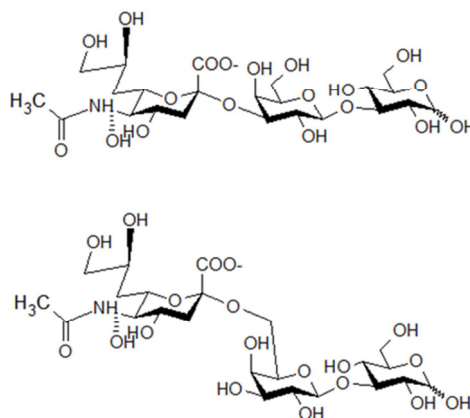
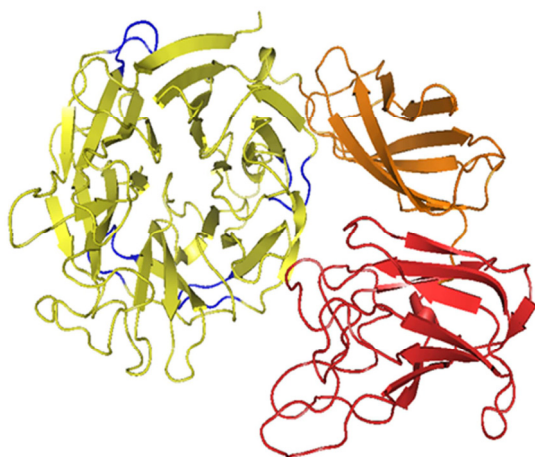
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Enzymatic production of human milk oligosaccharides



Yao Guo
Ph.D. Thesis
April 2014

Enzymatic production of human milk oligosaccharides



Yao Guo

Ph.D. thesis, April 2014

Center for BioProcess Engineering

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April 2014

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Preface

This thesis comprises the research carried out during my PhD study at the Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark, from August 2010 to April 2014.

The work was accomplished under the supervision of Professor Jørn D. Mikkelsen and co-supervised by Postdoc Carsten Jers, both from the Center for BioProcess Engineering.

The study was funded by a grant from the Danish Strategic Research Council (Enzymatic Production of Human Milk Oligosaccharides, 09-067134).

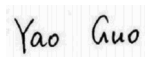
This thesis is submitted towards fulfilling the requirements for obtaining the degree of PhD at the Technical University of Denmark.

First of all, I would like to thank my supervisor Professor Jørn D. Mikkelsen for offering me the opportunity to pursue my PhD study, and for giving me valuable advices and challenges, endless encouragement and support throughout my PhD period. Special thanks go to my co-supervisor Postdoc Carsten Jers, always being friendly and patient to discuss every issue I bring. I want to thank you for showing me your discreet attitude towards research and for constantly guiding me with your inspiring thoughts. I would also like to thank Professor Anne S. Meyer for giving me constructive suggestions especially during the time of writing papers and thesis.

I would like to thank my fellows from the “enzymatic production of human milk oligosaccharides” project. Thanks go to Rui Xue for her valuable inputs in the beginning of the research, to Haiying Li and Finn Kirpekar for carrying out MS analysis, to Charlotte Held Gotfredsen and Louise Kjærulff for carrying out NMR analysis, to Malwina Michalak, Jesper Holck and Rune T. Nordvang for assisting me in purifying oligosaccharides, to Christian Nyffenegger for helping me with analysis of enzyme kinetics, to Jianquan Luo for helping me with membrane separation, and to Birgitte Zeuner for helping me with translation of the summary.

I would also like to thank my colleagues at the Center for Bioprocess Engineering, especially Tao Feng, Dorte M. Larsen, Mateusz Lezyk, Inês Rodrigues da Silva, Dayanand Kalyani, Søren Brander, and Guotao Sun for sharing good time with me, both inside and outside of the GMO lab. Thank Annette Eva Jensen and Anis Arnous for their excellent help with HPAEC analysis.

Last but not least, I want to thank my parents, younger brother, family and friends for their never-ending love and support.



Yao Guo

Nærum, April 2014

Summary

Enzymatic treatment of biomass is an environmentally friendly method to obtain a range of value-added products, such as biofuels, animal feed or food ingredients. The objective of this PhD study was to biocatalytically produce biofunctional food ingredients – human milk oligosaccharides decorated with sialic acid from casein glycomacropeptide obtained from dairy side streams. In addition, the biocatalysts employed in this study, *i.e.*, a sialyltransferase and a sialidase, were subjected to protein engineering to alter the enzyme's regioselectivity and to improve hydrolase activity, respectively.

A recombinant *Pasteurella multocida* sialyltransferase (EC 2.4.99.-), namely PmST, exhibiting promiscuous trans-sialidase activities was examined. The enzyme catalysed α -2,3- and α -2,6-sialylation of lactose using either 2-*O*-(*p*-nitrophenyl)- α -*D*-*N*-acetylneuraminic acid or casein glycomacropeptide as a sialyl donor. This is the first study reporting α -2,6-trans-sialidase activity of this enzyme. Using response surface design allowed identification of two differently optimised conditions for PmST-catalysed production of 3'-sialyllactose and 6'-sialyllactose, giving maximum yields of 2.8 mM and 3.3 mM from casein glycomacropeptide (9 mM bound sialic acid), respectively. The k_{cat}/K_m value for PmST catalysing 6'-sialyllactose synthesis using 3'-sialyllactose as donor was $23.2 \pm 0.7 \text{ M}^{-1} \text{ s}^{-1}$. Further, the enzyme was capable of catalysing synthesis of both 3'- and 6'-sialylated galactooligosaccharides with use of galactooligosaccharides as acceptors.

Secondly, we examined the regioselectivity of five designed mutants of PmST catalysing synthesis of 3'- and 6'-sialyllactoses using casein glycomacropeptide and lactose as substrates. The mutants PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y} preferentially catalysed synthesis of 3'-sialyllactose over 6'-sialyllactose. The best mutant PmST_{E271F/R313Y} for α -2,3-trans-sialylation gave a maximum 3'-sialyllactose yield of 4.5 mM from casein glycomacropeptide (9 mM bound sialic acid). Another mutant PmST_{P34H} displayed a distinct preference for 6'-sialyllactose synthesis throughout the reaction, though the total sialyllactose yield was consistently and significantly lower than that using the wild type enzyme. PmST_{P34H} had a 980-fold increase in α -2,6-sialyltransferase activity compared to the wild type enzyme, while its α -2,3-sialyltransferase activity was almost abolished. The k_{cat}/K_m value for PmST_{P34H} catalysing 6'-sialyllactose synthesis using 3'-sialyllactose as donor was $31.2 \text{ M}^{-1} \text{ s}^{-1}$. Moreover, both the wild type enzyme and PmST_{P34H} were capable of catalysing the hydrolysis and transfer of α -2,6 bound sialic acid.

While enzymatic synthesis of 3'-sialyloligosaccharides can be efficiently catalysed by notably *Trypanosoma cruzi* α -2,3-trans-sialidase, the synthesis of 6'-sialyloligosaccharides using cheap sialyl donor has been hampered by the lack of efficient enzymes. The combinatory study of mutagenesis and reaction conditions in this work has provided new tools for the efficient synthesis of 6'-sialyloligosaccharides.

The third part of the work aimed to improve the hydrolase activity of a sialidase from *Micromonospora viridifaciens* (EC 3.2.1.18) that can catalyse cleavage of α -2,3, α -2,6 and α -2,8 sialyl linkages. The enzyme and its mutants were expressed in *Bacillus subtilis* and secreted to the culture medium. Twenty amino acid residues within 10 Å of the sialic acid binding site were targeted for site-saturation mutagenesis and evaluated on the artificial substrate 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminic acid and the natural substrate casein glycomacropeptide. A much higher proportion of mutants displayed increased sialidase activity on the artificial substrate than on the natural one. The most proficient mutant showed a 27-fold increase in activity on the artificial substrate, whereas no mutants displayed more than a 2-fold activity increase on the natural one. By analysing the stability of mutants with use of the PoPMuSiC software, it was suggested that the enzyme almost reached proficiency optimum towards the natural substrate, while its increased activity on the artificial substrate correlated with major chemical variation and decreased stability, consistent with a trade-off between stability and activity as commonly observed in protein engineering.

Resumé

Enzymatisk behandling af biomasse er en miljøvenlig metode til at fremstille en række værdiskabende produkter, såsom biobrændsel, foder og fødevaringredienser.

Formålet med dette ph.d.-studie var at producere biofunktionelle fødevaringredienser enzymatisk – med fokus på humane mælkeoligosakkarider, der blev dekoreret med sialinsyre fra kasein glycomakropeptid som findes i en sidestrøm fra mejeriproduktionen. Desuden blev det forsøgt vha. mutagenese at ændre regioselektiviteten hos en sialyltransferase og at forbedre hydrolyseaktiviteten hos en sialidase.

PmST, en rekombinant *Pasteurella multocida* sialyltransferase (EC 2.4.99-), der har forskellige trans-sialidaseaktiviteter blev undersøgt. Både med 2-*O*-(p-nitrophenyl)- α -*D*-*N*-acetylneuraminsyre og kasein glycomakropeptid som sialyldonor var enzymet i stand til at katalysere α -2,3- og α -2,6-sialylering af laktose. Dette studie er det første, der demonstrerer α -2,6-trans-sialidaseaktivitet for dette enzym. Reaktionsbetingelserne for PmST-katalyseret syntese af 3'- og 6'-sialyllaktose blev optimeret vha. responsflade-design, og under de optimale betingelser var udbyttet hhv. 2.8 mM og 3.3 mM med kasein glycomakropeptid (9 mM bundet sialinsyre) som donor. For PmST-katalyseret syntese af 6'-sialyllaktose med 3'-sialyllaktose som donor var k_{cat}/K_m $23.2 \pm 0.7 \text{ M}^{-1} \text{ s}^{-1}$. Endvidere var enzymet i stand til at katalysere både α -2,3- og α -2,6-sialylering af galaktooligosakkarider.

Derudover undersøgte vi regioselektiviteten hos fem PmST-mutanter i forhold til syntese af 3'- og 6'-sialyllaktose med kasein glycomakropeptid og laktose som substrater. Mutanterne PmST_{E271F}, PmST_{R313Y} og PmST_{E271F/R313Y} katalyserede fortrinsvis syntese af 3'- sialyllaktose, hvor den bedste mutant PmST_{E271F/R313Y} gav et maksimalt udbytte af 3'-sialyllaktose på 4.5 mM med kasein glycomakropeptid (9 mM bundet sialinsyre) som donor. En anden mutant, PmST_{P34H}, udviste en klar præference for syntese af 6'-sialyllaktose syntese om end det samlede udbytte af sialyllaktose var konsekvent og signifikant lavere end ved anvendelse af vildtype-enzymet. Desuden udviste PmST_{P34H} en 980 gange højere α -2,6-sialyltransferase aktivitet end vildtype-enzymet, hvorimod α -2,3-sialyltransferase aktiviteten var svært reduceret. For PmST_{P34H}-katalyseret syntese af 6'-sialyllaktose med 3'-sialyllaktose som donor var k_{cat}/K_m $31.2 \text{ M}^{-1} \text{ s}^{-1}$. Desuden var både vildtype-enzymet og PmST_{P34H} i stand til at katalysere hydrolyse og overførsel af α -2,6-bundet sialinsyre.

Mens enzymatisk syntese af 3'-sialyloligosakkarider effektivt kan katalyseres af især *Trypanosoma cruzi* α -2,3-trans-sialidase, er syntese af 6'-sialyloligosakkarider under anvendelse af en billig

sialyldonor hæmmet af manglen på effektive enzymer. Den kombinerede undersøgelse af mutagenese og reaktionsbetingelser i dette studie har givet nye værktøjer til effektiv syntese af 6'-sialyloligosakkarider.

Endelig blev det forsøgt at forbedre hydrolyseaktiviteten for en sialidase fra *Micromonospora viridifaciens* (EC 3.2.1.18), der katalyserer hydrolyse af α -2,3-, α -2,6- og α -2,8-bindinger. Enzymet og mutanter deraf blev udtrykt i *Bacillus subtilis*, hvor det blev secerneret til vækstmediet. På baggrund af krystalstrukturen blev tyve aminosyrer inden for 10 Å af sialinsyrebindingsstedet udvalgt til 'site-saturation' mutagenese. Disse mutanter blev evalueret for hydrolaseaktivitet med det unaturlige substrat 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminsyre og det naturlige substrat kasein glycomakropeptid. Det blev fundet, at en langt større fraktion af mutanterne udviste øget hydrolyseaktivitet med det unaturlige substrat i forhold til det naturlige substrat. Den bedste mutant udviste 27 gange højere hydrolyseaktivitet med det unaturlige substrat, hvorimod ingen mutanter havde mere end to gange højere aktivitet med det naturlige substrat. Mutanternes stabilitet blev analyseret ved hjælp af PoPMuSiC algoritmen, og dette indikerede at sialidasen havde en nær-optimal aktivitet med det naturlige substrat, hvorimod øget aktivitet med det unaturlige substrat korrelerede med større kemisk variation og nedsat stabilitet. Dette er i overensstemmelse med en trade off mellem stabilitet og aktivitet som er blevet observeret i protein optimering.

List of publications

- I. Guo, Y., Jers, C., Meyer, A. S., Arnous, A., Li, H., Kirpekar, F., Mikkelsen, J. D. (2014) A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3'-sialyl and 6'-sialyl glycans. *Journal of Biotechnology* **170**, 60-67
- II. Guo, Y., Jers C., Meyer, A. S., Li, H., Kirpekar, F., Mikkelsen, J. D.. Modulating the regioselectivity of *Pasteurella multocida* sialyltransferase for biocatalytic production of 3'- and 6'-sialyllactose. Submitted.
- III. Jers, C., Guo, Y., Kepp, K. P., Mikkelsen, J. D.. Mutants of *Micromonospora viridifaciens* sialidase have highly variable activities on natural and non-natural substrates. Submitted to Protein Engineering, Design and Selection.

Publications not included in this thesis

- I. Jers, C., Michalak, M., Larsen, D. M., Kepp, K. P., Li, H., Guo, Y., Kirpekar, F., Meyer, A. S., Mikkelsen, J. D. (2014) Rational design of a new *Trypanosoma rangeli* trans-sialidase for efficient sialylation of glycans. *Plos One* **9**, e83902
- II. Li, H., Rasmussen, M., Larsen, M.R., Guo, Y., Jers, C., Palmisano, G., Mikkelsen, J.D., Kirpekar, F.. Automated N-glycan profiling of a mutant *Trypanosoma rangeli* sialidase, expressed in *Pichia pastoris*, using tandem mass spectrometry and bioinformatics. Submitted to Glycobiology.

Abbreviations

ATP	adenosine-5'-triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAZY	carbohydrate-active enzymes
cGMP	casein glycomacropeptide
CMP-Neu5Ac	cytidine-5'-monophosphate- <i>N</i> -acetylneuraminic acid
CMP-3F(e)Neu5Ac	cytidine-5'-monophosphate-3-equatorial-fluoro- <i>N</i> -acetylneuraminic acid
CTP	cytidine-5'-triphosphate
CV	column volume
DANA	2-deoxy-2,3-dehydro- <i>N</i> -acetylneuraminic acid
Fuc	fucose
FUT	fucosyltransferase
Gal	galactose
GalNAc	<i>N</i> -acetylgalactosamine
GalT	galactosyltransferase
GH	glycoside hydrolase
Glc	glucose
GlcNAc	<i>N</i> -acetylglucosamine
GNE	uridine-5'-diphosphate- <i>N</i> -acetylglucosamine-2-epimerase/ <i>N</i> -acetylmannosamine kinase
GlcNAcT	<i>N</i> -acetylglucosaminyltransferase
GOS	galactooligosaccharides
GMP	guanosine-5'-monophosphate
GT	glycosyltransferase
GTP	guanosine-5'-triphosphate
HMOs	human milk oligosaccharides
HPAEC-PAD	high-performance anion exchange chromatography with pulsed amperometric detection
KDN	3-deoxy- <i>D</i> -glycero- <i>D</i> -galacto-2-nonulopyranos-1-onic acid
κ-CN	κ-casein
LC-MS	capillary liquid chromatography/mass spectrometry
LNac	<i>N</i> -acetylglucosamine
LNDFH	lacto- <i>N</i> -difucohexaose
LNnDFH	lacto- <i>N</i> -neodifucohexaose
LNFP	lacto- <i>N</i> -fucopentaose
LNnFP	lacto- <i>N</i> -neofucopentaose
LNnH	lacto- <i>N</i> -neohexaose

LNT	lacto- <i>N</i> -tetraose
LNnT	lacto- <i>N</i> -neotetraose
LOS	lipooligosaccharide
LST	sialyl-lacto- <i>N</i> -tetraose
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
Man	mannose
ManNAc	<i>N</i> -acetylmannosamine
Mu-Neu5Ac	4-methylumbelliferyl- <i>N</i> -acetyl- α - <i>D</i> -neuraminic acid
N-His ₆ -tag	N-terminal His ₆ -tag
Neu5Ac	<i>N</i> -acetylneuraminic acid
Neu5Gc	<i>N</i> -glycolylneuraminic acid
neuraminic acid	5-amino-3,5-dideoxy- <i>D</i> -glycero- <i>D</i> -galacto-nonulopyranos-1-onic acid
NMR	nuclear magnetic resonance spectroscopy
PEP	phosphoenolpyruvate
Ph- β -Neu5Ac	phenyl- β -Neu5Ac
PmST	<i>Pasteurella multocida</i> sialyltransferase
pNP-Neu5Ac	2- <i>O</i> -(p-nitrophenyl)- α - <i>D</i> - <i>N</i> -acetylneuraminic acid
RSM	response surface methodology
SAS	Neu5Ac synthase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sia-GOS	sialylated galactooligosaccharides
SiaT	sialyltransferase
SL	sialyllactose
SLN	sialyl- <i>N</i> -acetylglucosamine
TcTS	<i>Trypanosoma cruzi</i> trans-sialidase
TLC	thin-layer chromatography
TrSA	<i>Trypanosoma rangeli</i> sialidase
TrSA ₅	<i>Trypanosoma rangeli</i> sialidase with 5 mutations
TrSA ₆	<i>Trypanosoma rangeli</i> sialidase with 6 mutations
TrSA ₁₃	<i>Trypanosoma rangeli</i> sialidase with 13 mutations
U	unit
UDP-GlcNAc	uridine-5'-diphosphate- <i>N</i> -acetylglucosamine
UTP	uridine-5'-triphosphate

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1. INTRODUCTION

1.1 HUMAN MILK OLIGOSACCHARIDES (HMOs)

1.1.1 Structure and biosynthesis of HMOs

Oligosaccharides are the third largest abundant component in human milk after lactose and fat. The content of HMOs varies during lactation with a level of >20 g/L in colostrum and 5-14 g/L in mature milk (Coppa et al., 1999; Gabrielli et al., 2011; Kunz et al., 2000). The building blocks of HMOs comprise five monosaccharides, *i.e.* glucose (Glc), galactose (Gal), fucose (Fuc), *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (Neu5Ac). Lactose (Gal β -1,4Glc) forms the reducing end of HMOs. Gal in lactose can be sialylated in α -2,3 and α -2,6 linkages to form 3'- and 6'-sialyllactoses (SLs) (Fig. 1.1), or fucosylated in α -1,2 and α -1,3 linkages to form 2'-fucosyllactose (2'-FL, Fuc α 1-2Gal β 1-4Glc) and 3-FL (Gal β 1-4(Fuc α 1-3)Glc), respectively. These trisaccharides constitute the short chain HMOs. To form more complex structures of HMOs, lactose is elongated with *N*-acetylglucosamine (LNac) repeat units (Gal β 1-3/4GlcNAc). Lactose and/or polylactosamine backbone can be further sialylated and/or fucosylated in various linkages (Bode, 2009). About 180 different oligosaccharide species have been identified from a pooled human milk sample, containing nearly 46% fucosylated and 17% sialylated oligosaccharides (Niñonuevo et al., 2006). Conversely, bovine milk contains no neutral oligosaccharides *e.g.* lacto-*N*-tetraose (LNT, Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc) and fucosylated oligosaccharides but mainly sialylated oligosaccharides with a 20-fold lower level compared to the human milk counterpart (Table 1.1).

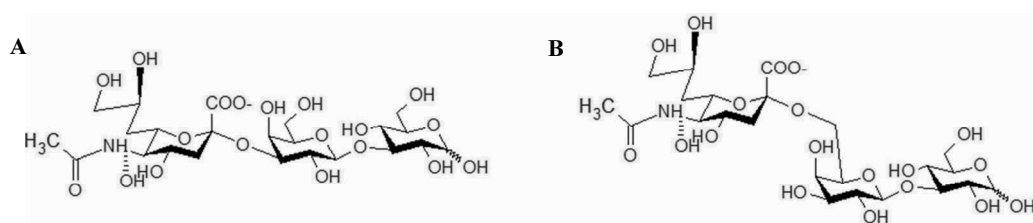


Fig. 1.1 Structures of 3'-SL (A) and 6'-SL (B).

The chemical structures of HMOs have been studied using reversed-phase high performance liquid chromatography (RP-HPLC) (Chaturvedi et al., 1997; Leo et al., 2010; Sumiyoshi et al., 2003), high-performance anion exchange chromatography (HPAEC) (Kunz et al., 1996; Finke et al., 1999; Thurl et al., 1996), capillary electrophoresis (CE) (Bao and Newburg, 2008; Shen et al., 2000),

associated with nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) techniques (Kogelberg et al., 2004; Niñonuevo et al., 2008; Stahl et al. 1994; Wu et al., 2011). Particularly, a method using matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS) allowed precise identification and quantification of individual HMO species (Niñonuevo et al., 2006; LoCascio et al., 2009).

Table 1.1 Major oligosaccharides of human milk and bovine milk^a.

Oligosaccharide	Human milk (g/L) ^b	Bovine milk (g/L) ^c
Lacto- <i>N</i> -tetraose	0.5-1.5	-
Lacto- <i>N</i> -fucopentaose I	1.2-1.7	-
Lacto- <i>N</i> -fucopentaose II	0.3-1.0	-
Lacto- <i>N</i> -fucopentaose III	0.01-0.2	-
Lacto- <i>N</i> -difucohexaose	0.1-0.2	-
6'-sialyllactose	0.3-0.5	0.03-0.06 ^d
3'-sialyllactose	0.1-0.3	
Neu5Ac-lacto- <i>N</i> -tetraose a	0.03-0.2	Traces
Neu5Ac-lacto- <i>N</i> -tetraose c	0.1-0.6	Traces
Neu5Ac ₂ -lacto- <i>N</i> -tetraose	0.2-0.6	Traces
In total	5.0-8.0	Traces

^a Table is adapted from Kunz et al. (2000).

^b Data are obtained from Kunz and Rudloff (1993) and Montreuil et al., 1960.

^c Data are obtained from Parkkinen and Finne (1987) and Martín-Sosa et al. (2003).

^d The value denotes the total concentration of 3'- and 6'-SLs.

Biosynthesis of HMOs is suggested to be an extension of lactose synthesis that occurs in the Golgi (Bode, 2012). It starts with activation of cytosolic Glc to UDP-Glc which is then converted to UDP-Gal. Rudloff et al. (2006) suggested that exogenous Gal might directly be incorporated to lactose and HMOs without prior conversion to Glc and reconversion to Gal. Eventually, both UDP-Gal and Glc are transported into the Golgi and are linked to the lactose synthase complex that consists of “A” and “B” proteins. In combination with “B” protein α -lactalbumin, “A” protein β -1,4-galactosyltransferase (β -1,4GalT) shifts its acceptor specificity from GlcNAc to Glc and thus catalyses the transfer of UDP-Gal to Glc to form lactose (Ramakrishnan et al., 2002). The biosynthetic steps for extending lactose to form different HMOs remain poorly understood. However, Kobata (2003) suggested that extension of the lactose core might be fulfilled by concerted action of *N*-acetylglucosaminyltransferases (GlcNAcT) and GalT that enable synthesis of two major tetrasaccharide structures LNT and lacto-*N*-neotetraose (LNnT, Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc). HMO fucosylation involves three fucosyltransferases (FUTs), *i.e.* FUT2 catalyses

the transfer of α -1,2 bound Fuc to terminal Gal, FUT3 catalyses the transfer of α -1,4 bound Fuc to internal GlcNAc preferentially on type 1 chains (Gal β 1-3GlcNAc-), and FUTX (an unknown FUT) catalyses the transfer of α -1,3 bound Fuc to terminal Glc or subterminal GlcNAc on type 2 chains (Gal β 1-4GlcNAc-) (Chaturvedi et al., 2001; Gabrielli et al., 2011; Kumazaki and Yoshida, 1984; Johnson and Watkins, 1992; Stahl et al., 2001; Thurl et al., 1997). Little knowledge has been obtained for sialyltransferase (SiaT) types that are responsible for HMO sialylation. However, Tsuchida et al. (2003) reported that two α -2,6-SiaTs (namely ST6GalNAc V and VI) were able to facilitate α -2,6-sialylation of subterminal GlcNAc.

1.1.2 Physiological properties of HMOs

The structural diversity and complexity confer unique biological functions to HMOs. Firstly, HMOs serve as prebiotics that promote the growth of desired bacteria in the intestine of infant. For example, a mixture of ~10 HMOs containing GlcNAc is the growth-promoting “bifidus factor” for *Bifidobacterium bifidum* (György et al., 1954; Petschow and Talbott, 1991). Short-chain HMOs (degree of polymerization <7) are preferentially consumed by *Bifidobacterium longum* subsp. *infantis* (LoCascio et al., 2007 and 2009). Metabolites from bacterial degradation of HMOs, e.g., short-chain fatty acids and lactic acid, create an environment that favors the growth of commensals over potential pathogens (Gibson and Wang, 1994; Ogawa et al., 1992). Recently, sequencing of the *B. infantis* genome revealed a 43 kb gene cluster encoding glycosidases (sialidase, fucosidase, galactosidase and hexosaminidase), oligosaccharide transport-related genes as well as glycan-binding proteins that are responsible for HMO metabolism (Sela et al., 2008). Secondly, HMOs act as “decoys”, namely bacterial lectin ligand analogues, to block pathogen attachment (Newburg et al., 2005). Parkkinen et al. (1983) reported that sialylated HMOs abolished the binding activity of *Escherichia coli* strains that cause newborn meningitis. The α -1,2-fucosylated HMOs might reduce the diarrhea incidence by preventing *Campylobacter jejuni* from binding to human epithelial surface (Ruiz-Palacios et al., 2003; Morrow et al., 2004). Fucosylated HMOs might also reduce human immunodeficiency virus (HIV)-1 mother-to-child transmission by competing with the viral envelope glycoprotein (gp120) for binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (Hong et al., 2009). The antiadhesive effect of HMOs also applies to protozoan parasites like *Entamoeba histolytica* that causes amoebic dysentery or amoebic liver abscess (Jantscher-Krenn et al., 2012). Thirdly, HMOs can modify the intestinal epithelial cell surface glycome to impact pathogen attachment. Angeloni et al. (2005) reported that 3'-SL-induced changes

in the cell-surface glycans led to a 90% reduction in enteropathogenic *E. coli* adhesion by regulating the expression of genes that encode enzymes involved in glycan assembly. Fourthly, HMOs directly affect the infant immune system by altering protein-carbohydrate interactions at a systemic level. For instance, Bode et al. (2004a and 2004b) reported that sialylated HMOs might serve as anti-inflammatory components to lower the incidence of inflammatory diseases by affecting leukocyte-endothelial cell and leukocyte-platelet interactions. Lastly, Neu5Ac and Fuc moieties derived from degraded HMOs are readily available for synthesis of biologically active substances such as gangliosides and cerebral glycoproteins involved in neuronal growth and morphology, and might therefore improve memory-storage and learning ability (Krug et al., 1994; Matthies et al., 1996; Wang, 2009; Wang et al., 2003).

1.2 SIALIC ACID

Sialic acids are a family of α -keto acids with a nine-carbon backbone. Over 50 sialic acid forms have been elucidated, which are derivatives of neuraminic acid (5-amino-3,5-dideoxy-*D*-glycero-*D*-galacto-nonulopyranos-1-onic acid). The structural modification of sialic acids generally occurs at carbon 5, *i.e.*, the C-5 position commonly has an acetamido giving Neu5Ac, or a hydroxyacetamido giving *N*-glycolylneuraminic acid (Neu5Gc), or a hydroxyl moiety giving 3-deoxy-*D*-glycero-*D*-galacto-2-nonulopyranos-1-onic acid (KDN) (Fig. 1.2). These three molecules (Neu5Ac, Neu5Gc and KDN) can be further modified by substitutions at the hydroxyl groups on C-4, C-7, C-8 and C-9 with *O*-acetyl, *O*-methyl, *O*-sulfate, and lactyl and phosphate groups. The carboxylate group of sialic acids is deprotonated at physiological pH (pKa of 2.6) and confers the net negative charge that dominates physiological properties of the family (Schauer, 1997 and 2004; Varki, 1999; Vimr et al., 2004).

Sialic acids usually occupy the terminal position of cell surface-exposed glycoconjugates and are involved in many biological and pathological processes, including cell-cell and cell-molecule interactions. Due to their negative charge, sialic acids are involved in the binding and transport of positively charged molecules (*e.g.* Ca^{2+}) as well as in the repulsion of cells, *e.g.* erythrocytes (antiadhesive effect), and influence the physicochemical properties of glycoproteins such as mucins. The negative charge of sialic acids also protects glycoproteins from enzymatic, mainly proteolytic attack. Sialic acids play the most important role as a mask to prevent biological recognitions, or in opposite, to represent recognition determinants. For instance, by shielding recognition sites such as penultimate monosaccharides or antigenic proteins, sialic acids often render cells as “self” to avoid

or resist host innate immunity; as well, sialic acids serve as receptors in the adhesion of pathogenic viruses, bacteria, and protozoa; and they are also believed to regulate cell adherence and mobility during embryogenesis and malignant growth (Schauer, 2000a and b and 2009; Traving and Schauer, 1998).

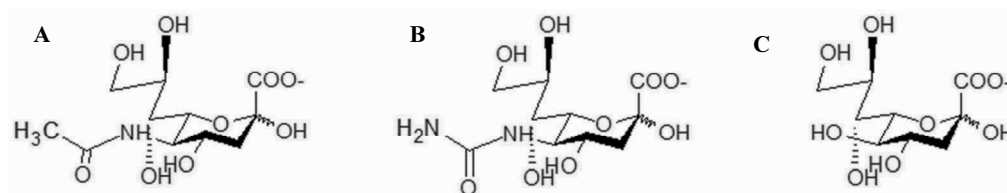


Fig. 1.2 Chemical structures of three naturally abundant sialic acids. (A) Neu5Ac; (B) Neu5Gc; (C) Kdn.

De novo biosynthesis of sialic acid differs in eukaryotes and bacteria. In most eukaryotes, it takes place in cytosol involving three enzymes in a four-step process. The first two steps are catalysed by a bifunctional enzyme uridine-5'-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc)-2-epimerase/*N*-acetylmannosamine (ManNAc) kinase (named GNE), *i.e.*, the UDP-GlcNAc-2-epimerase activity of GNE catalyses the conversion of UDP-GlcNAc to ManNAc; the kinase function of GNE subsequently phosphorylates ManNAc to form ManNAc-6-P. The latter two steps comprise condensation and dephosphorylation reactions catalysed by Neu5Ac 9-phosphate synthase (NANS) and Neu5Ac-9-phosphate phosphatase (NANP) respectively to produce sialic acid. Only a limited number of pathogenic bacteria and commensals which are mostly related to human are able to synthesize sialic acid. *De novo* biosynthesis of sialic acid in bacteria begins with the conversion of UDP-GlcNAc to ManNAc catalysed by UDP-GlcNAc-2-epimerase. ManNAc is then converted to sialic acid and inorganic phosphate catalysed by Neu5Ac synthase (SAS) in the presence of phosphoenolpyruvate (PEP). Sialic acid activation and transfer processes are conserved from bacteria through humans. In eukaryotes, sialic acid synthesized in the cytosol is transferred to the nucleus and activated by cytidine-5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) synthetase to form CMP-Neu5Ac, which is then transferred to the Golgi and used by SiaTs for the formation of sialoglycoconjugates. Degradation of sialoglycoconjugates is carried out in lysosome by sialidases, and released sialic acid go back into the cytosol and are subjected to another cycle of sialoglycoconjugate production, or are broken down by sialic acid lyase to form ManNAc and pyruvate. Similarly, sialylation in bacteria is mainly catalysed by SiaTs; as well, desialylation is carried out by sialidases or sialic acid lyase (Angata and Varki, 2002; Buschiazzi and Alzari, 2008; Li and Chen, 2012; Traving and Schauer, 1998).

Exceptionally, some protozoan species, such as *Trypanosoma cruzi* (the causative agent of Chagas' disease), use an α -2,3-trans-sialidase (TcTS) to catalyse the transfer of sialic acid residues directly from the hosts to terminal β -Gal residues of the parasite mucins and form their own surface sialoglycoconjugates (Colli, 1993; Previato, 1985). A few bacterial pathogens have evolved scavenging pathways to obtain sialic acids, including donor scavenging (only described in *Neisseria gonorrhoeae*) where CMP-sialic acid is scavenged from the hosts (Parsons et al., 1994), and precursor scavenging (e.g., *Haemophilus influenzae* and *Pasteurella multocida*) where free sialic acid is obtained directly from the hosts (Schilling et al., 2001; Steenbergen et al., 2005).

1.3 BACTERIAL SIALIDASE

Sialidases, or neuraminidases (EC 3.2.1.18) belong to a class of glycoside hydrolases (GHs) that catalyse the release of terminal sialic acid residues from a variety of sialoglycoconjugates in diverse organisms, including animals, microorganisms and viruses. Sialidases may be extracellular, membrane-bound or intracellular in bacteria. The enzymes are used to scavenge sialic acids as carbon and energy sources from sialylated substrates, or function as virulence factors that contribute to the recognition of sialic acids exposed on the cell surface of the hosts (Corfield, 1992; Roggentin et al., 1993; Taylor, 1996; Vimr, 1994).

1.3.1 Structural and biochemical properties

The monomeric molecular weights of bacterial sialidases generally range between 40 kDa and 150 kDa. Most of the enzymes are monomers, whereas sialidases from *Clostridium chauvoei* (dimer), *Bacteroides fragilis* SBT3182 (trimer) and *Trichomonas fetus* (tetramer) are exceptions (Table 1.2). Bacterial sialidases can be divided into small and large subtypes on the basis of molecular weight, i.e., small sialidases possess only a catalytic domain and large sialidases include additional domains that assist with carbohydrate-binding and enzyme location. *Micromonospora viridifaciens* secretes a small or large sialidase derived from the same gene, dependent on the carbon source: in the presence of colominic acid and milk casein, the small form of sialidase domain (41 kDa) and the large form (68 kDa) with an immunoglobulin fold and a Gal-binding domain following the catalytic domain are secreted, respectively (Fig. 1.3A) (Gaskell et al., 1995; Sakurada et al., 1992). The overall amino acid sequence homologies among bacterial sialidases are less than 30%, nevertheless, the catalytic domains share a common six-bladed β -propeller fold, in which each blade is made from a four-stranded antiparallel β -sheet (Fig. 1.3A). The enzymes usually contain three to five

Table 1.2 Biochemical properties of bacterial sialidases.

Enzyme source	Molecular weight (kDa)	Cell localization	Optimum pH	Optimum T (°C)	K _m (SL) mM	Linkage preference	Reference
<i>Actinomyces pyogenes</i>	50	Extracellular	6.0	55	0.14	α -2,3; α -2,6; α -2,8	Schaufuss and Lammlier, 1989
<i>Actinomyces viscosus</i>	150	Extracellular, membrane-bound	5.0	37	3.3-4.9	α -2,6 > α -2,8 > α -2,3	Teufel et al., 1989
<i>Aerobacter ureafaciens</i>	L	Extracellular	4.5-6.0	50	0.8	α -2,6 > α -2,3 > α -2,8	Ohta et al., 1989
	M1	Extracellular	4.5-6.0		0.6		
	M2	Extracellular	4.5-6.0		0.7		
	S	Extracellular	4.0-5.5		0.6		
<i>Bacteroides fragilis</i> 4852	180	Extracellular	6.0	-	1.6-2.9	α -2,3 > α -2,8 > α -2,6	von Nicolai et al., 1983
<i>Bacteroides fragilis</i> SBT3182	165 ^a (55) ^b	Extracellular	6.1	-	1.2-1.5	α -2,8 > α -2,3 > α -2,6	Tanaka et al., 1992 and 1994
<i>Bifidobacterium bifidum</i>	87	Extracellular, membrane-bound	4.0-5.0	50	0.028 ^c	α -2,3 > α -2,8 > α -2,6	Kiyohara et al., 2011
<i>Clostridium chauvoei</i>	300 ^a (150) ^b	Extracellular	5.5	37	2.0-2.2	α -2,3 > α -2,6 > α -2,8	Heuermann et al., 1991
<i>Clostridium Perfringens</i>	NanH	Intracellular	6.1	37	0.53-3.5	α -2,3 > α -2,6 > α -2,8	Kruse et al., 1996
	NanI	Extracellular	5.0	55	0.56-1.7		Peter et al., 1995
<i>Corynebacterium diphtheriae</i>	66	Extracellular, membrane-bound, cytoplasm	7.5	-	5.5-7.0	α -2,3; α -2,6	Kim et al., 2010
<i>Erysipelothrix rhusiopathiae</i>	48	Extracellular	6.2	-	0.9-5	α -2,3 > α -2,6 > α -2,8	von Niholai et al., 1978
<i>Micromonospora viridifaciens</i>	41/68	Extracellular	5.0	58	2.1	α -2,3 > α -2,8 > α -2,6	Aisaka and Uwajima, 1987; Aisaka et al., 1991
<i>Pasteurella multocida</i>	80	Extracellular	6.2-6.8	-	0.063 ^c	α -2,3 > α -2,6 > α -2,8	Stallings et al., 2000
<i>Salmonella typhimurium</i>	42	Extracellular	5.5-7.0	-	0.25 ^c	α -2,3 > α -2,6 > α -2,8	Hoyer et al., 1991
<i>Streptococcus pneumoniae</i>	NanA	Membrane-bound	6.5-7.0	-	-	α -2,3; α -2,6	Berry et al., 1996;
	NanB	Extracellular	4.5	-	-	α -2,3	Canara et al., 1994; Gut et al., 2008
<i>Tannerella forsythia</i>	57	Extracellular	5.5	-	0.033 ^c	α -2,3 > α -2,6 > α -2,8	Thompson et al., 2009
<i>Streptomyces griseus</i> MB395-A5	32	Extracellular	3.5-5.0	-	0.4	α -2,3 > α -2,6 > α -2,8	Kunimoto et al., 1974
<i>Streptomyces griseus</i> MB503-C1					0.15		
<i>Trichomonas fetus</i>	I	Extracellular, membrane-bound	4.7-5.5	-	9.5-15	α -2,3 > α -2,6 > α -2,8	Crampen et al., 1979
	II				5.9-8.5		
<i>Vibrio cholerae</i>	90	Extracellular	4.9-5.6	-	1.2	α -2,3; α -2,6	Ada et al., 1961; Pye and Curtain, 1961

^a and ^b oligomer and monomer forms

^c The substrate was 4-methylumbelliferyl-L-N-acetyl- α -D-neuraminic acid (Mu-Neu5Ac).

copies of an Asp-box (S/T-X-D-X-G-X-T-W/F, X representing any amino acid), a surface-exposed turn located between the third and fourth strands of each sheet. These Asp-boxes occupy topologically equivalent positions in the fold, and are remote from the active site, suggesting they are probably not involved in catalysis. Another conserved motif “RIP”, which has variations like “FRIP”, “YRIP” or “DRIP”, contains one of the catalytic triad of Arg residues that interacts with the carboxyl group of the sialic acid substrate (Kim et al., 2011; Roggentin et al., 1993; Taylor et al., 2008; Vimr, 1994). The active site of the enzymes also contains a nucleophile pair of Tyr/Glu working as a charge-relay system where the Tyr residue performs the direct nucleophile attack, and an Asp residue that acts as the acid/base catalyst (Fig. 1.3B).

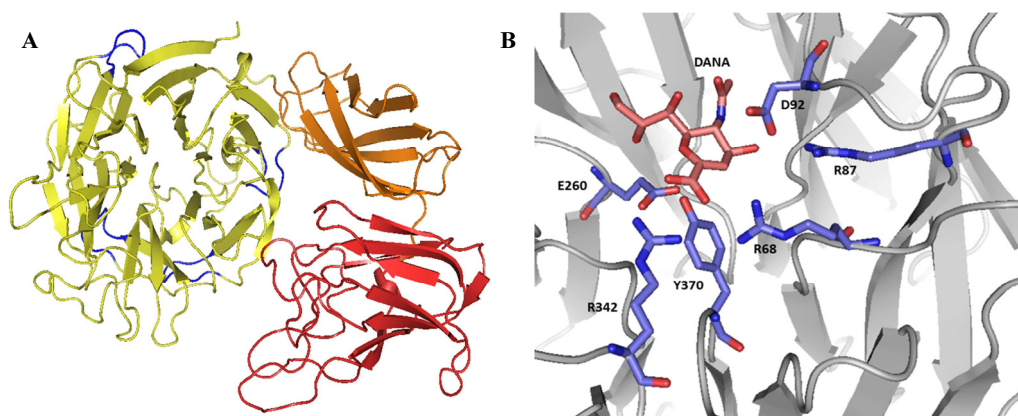


Fig. 1.3 Structure of *Micromonospora viridifaciens* sialidase (PDB code 1EUU). (A) Overall structure of the large form of the enzyme (68 kDa). The catalytic domain (41 kDa) is shown in yellow, the immunoglobulin fold in orange and the Gal-binding domain in red. The four Asp boxes in the catalytic domain are highlighted in blue. (B) Close view of the active site of the enzyme. The carbon atoms of the conserved amino acid residues and the ligand 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en, DANA) are shown in blue- and salmon-colored sticks, respectively.

Retaining bacterial sialidases, grouped in carbohydrate-active enzymes (CAZY) family GH 33, work through a classical ping-pong (double displacement) mechanism involving glycosylation and deglycosylation steps (Fig. 1.4). In the first step, with the aid of Glu the nucleophile (Tyr) attacks the anomeric carbon to displace the aglycon and form a covalent sialyl-enzyme intermediate, while the catalytic acid (Asp) protonates the glycosidic oxygen as the bond cleaves. In the second step, a water molecule is deprotonated by the catalytic base (Asp) in order to attack the anomeric center of

the sialyl-enzyme intermediate and restore the nucleophile Tyr (Buschiazzi and Alzari, 2008; Damager et al., 2008; Brás et al., 2012; Newstead et al., 2008; Watts et al., 2003).

The optimum temperatures of characterized bacterial sialidases are in the range of 37-58 °C, and the optimum pH values generally lie in a weakly acidic range (4.5-7.0). The kinetic properties can be affected significantly by difference in assay conditions. In general, the K_m values with SL as the substrate range between 0.5 mM and 5 mM (Table 1.2). Bacterial sialidases are promiscuous in their choice of sialoglycoconjugates, capable of hydrolysing either α -2,3, α -2,6, or α -2,8 bound sialic acid. In general, they preferentially hydrolyse 3'- or 6'-SL over colominic acid (polysialic acid with α -2,8 linkage), exceptions being sialidases from *Actinomyces viscosus*, *B. bifidum*, *B. fragilis* and *M. viridifaciens*. Most bacterial sialidases have a preference for 3'-SL rather than 6'-SL, especially *Streptococcus pneumoniae* sialidase is highly specific to α -2,3 sialyl linkage. *A. ureafaciens*, *A. viscosus* and *M. viridifaciens* sialidases, however, cleave α -2,6 bound sialic acid residues more efficiently than α -2,3 sialyl linkage (Table 1.2) (Abrashev and Dulguerova, 2000; Corfield, 1992; Kim et al., 2011; Roggentin et al., 1993; Saito and Yu, 1995).

1.3.2 Bacterial sialidase-catalysed trans-sialylation for sialyloligosaccharide synthesis

Conventional chemical synthesis remains a challenging task for production of sialyloligosaccharides because it involves multiple protection/deprotection and purification steps to achieve regioselectivity and stereoselectivity. To overcome these limitations, sialidase-catalysed trans-sialylation has been applied as an alternative for sialyloligosaccharide production (Desmet and Soetaert, 2011; Kim et al., 2011; Brás et al., 2012). The trans-sialylation reactions work through the double displacement mechanism of retaining sialidases, in which the covalent sialyl-enzyme intermediate is intercepted by an acceptor molecule other than water (Fig. 1.4). By use of appropriate selection of enzyme and reaction conditions, sialidases can catalyse highly regio- and stereo-specific bond formation.

The use of bacterial sialidases from *e.g.* *A. ureafaciens*, *B. fragilis*, *C. perfringens*, *Salmonella typhimurium*, and *Vibrio cholerae* as catalysts for synthesis of sialyloligosaccharides has been reported (Table 1.3). 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminic acid (pNP-Neu5Ac) is more commonly used as a sialyl donor compared to other sialoglycoconjugates because the leaving group pNP can be rapidly and irreversibly released by the enzyme during the glycosylation step (Kim et al., 2011; Seeberger et al., 2009). Bacterial sialidases catalyse preferentially formation of α -

2,6 sialyl linkage over α -2,3 linkage, except that *S. typhimurium* sialidase displays a distinct preference for α -2,3-trans-sialylation. Usually, bacterial sialidase-catalysed trans-sialylation reactions are carried out under mild conditions (pH 5.0-5.5, temperature 26-37 °C, in some cases with 10-30% co-solvent), and most reactions proceed at least for 5 h. The yields are generally low (<30%) because the products also undergo sialidase-catalysed hydrolysis. In order to drive the reactions towards the direction of trans-sialylation rather than hydrolysis, the use of high acceptor:donor ratio has been attempted with success.

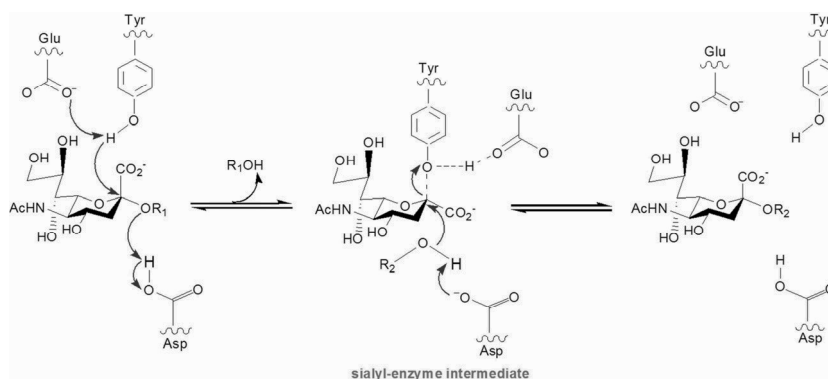


Fig. 1.4 Proposed catalytic mechanism for retaining sialidases. R₁, glycoside; R₂, glycoside or H.

Regioselectivity of glycosidase in transglycosylation reaction is highly correlated to its specificity in hydrolysis reaction (Ajisaka and Yamamoto, 2002). This rule applied to *S. typhimurium* sialidase that is highly specific for catalysing both transfer and hydrolysis of α -2,3 sialyl linkage. However, several exceptions have been found, *e.g.*, both *V. cholerae* and *C. perfringens* sialidases catalyse preferable formation of α -2,6 sialyl linkage over α -2,3 linkage, but their preference is reversed in hydrolysis reactions. One rationale for the regioselectivity difference between trans-sialylation and hydrolysis reactions could be that α -2,3-sialyloligosaccharides are rapidly hydrolysed and thus do not accumulate in the trans-sialylation reactions. Furthermore, regioselective trans-sialylation can also be controlled by the following factors: (1) Donor specificity. When the donor pNP-Neu5Ac was replaced with colominic acid or Neu5Aca2-8Neu5Ac, *V. cholerae* and *C. perfringens* sialidases catalysed formation of α -2,6 sialyl linkage rather than a mixture of isomers (Ajisaka et al., 1994). (2) Aglycon structure of acceptor. By use of the acceptor methyl α -D-galactopyranoside (α Gal-OCH₃), the regioisomeric ratio of (α -2,6: α -2,3) for *V. cholerae* sialidase increased 2-3 times compared to the corresponding ratios obtained by use of other galactoside acceptors (Thiem and Sauerbrei, 1991). The ratio between α -2,6- and α -2,3-sialylated products also varied depending on the acceptor

Table 1.3 Sialyloligosaccharides synthesis by bacterial sialidase-catalysed trans-sialylation.

Enzyme source	Donor	Acceptor	Donor/acceptor ratio	Reaction condition	Product ratio (α -2,3: α -2,6)	Transfer ratio ^c	Total yield (%) ^d	Reference
<i>A. ureafaciens</i>	Neu5Ac α 2-8Neu5Ac pNP-Neu5Ac	Gal β 1-4Glc	1:10 ^a	37 °C, pH 5.0, 5 h	0:100	5.6:94.4	-	Ajisaka et al., 1994
		Gal β 1-4Glc	1:4 ^a	37 °C, pH 5.0, 8 h, 30% ACN	0:100	7.4:92.6	-	
<i>B. fragilis</i>	Colomine acid	Gal β 1-4Glc	1:1 ^a	37 °C, pH 5.5, 3 h	1:68.3	-	29.1 ^e	Tanaka et al., 1995
<i>C. perfringens</i>	Colomine acid	Gal β 1-4Glc	1:10 ^a	37 °C, pH 5.5, 20 h	1:5.9	-	31.2 ^e	Ajisaka et al., 1994
				37 °C, pH 5.0, 5 h	0:100	2.4:97.6	0.8 ^e	
	Neu5Ac α 2-8Neu5Ac	Gal β 1-4Glc	1:10 ^a	37 °C, pH 5.0, 5 h	0:100	8.5:91.5	2.5 ^e	-
	Neu5Ac α 2-8Neu5Ac	Gal β 1-4GlcNAc	1:10 ^a	37 °C, pH 5.0, 5 h	0:100	5.5:94.5	-	
	pNP-Neu5Ac	Gal β 1-4Glc	1:4 ^a	37 °C, pH 5.0, 8 h, 30% ACN	30:70	12.4:87.6	-	
<i>S. typhimurium</i>	pNP-Neu5Ac	Tn-antigen	1:5 ^b	37 °C, pH 5.1, 24 h	95:5	-	15	Schmidt et al., 2000
		T-antigen			93:7		11	
		T-antigen precursor			88:12		11	
		α Gal-OCH ₃			95:5		16	
<i>V. cholerae</i>	pNP-Neu5Ac	β Gal-OCH ₃			99:1		15	Makimura et al., 1998 Thiem and Sauerbrey, 1991
		Le ^x	1:12.7 ^b	37 °C, pH 5.0, 2 h, 10% ACN	100:0	-	9.3	
		Le ^s			100:0		12.0	
		β Gal-OCH ₃	1:7 ^b	26 °C, pH 5.5, 41 h	1:2.9	-	20	
		β Gal-OCH ₃		26 °C, pH 5.5, 120 h, 30% DMSO	1:2.0		20	
		α Gal-OCH ₃		26 °C, pH 5.5, 45 h	1:6.0		24	
		β Gal-OCH ₃		26 °C, pH 5.5, 45 h	1:3.2		16	
		Gal β 1-4Glc-OCH ₃		26 °C, pH 5.5, 45 h	1:2.0		16	
		Gal β 1-4GlcNAc-OCH ₃		26 °C, pH 5.5, 68.5 h	1:2.8		14	
	Neu5Ac α 2-8Neu5Ac pNP-Neu5Ac	Gal β 1-4Glc	1:10 ^a	37 °C, pH 5.0, 5 h	0:100	4.1:95.9	-	
		Gal β 1-4Glc	1:4 ^a	37 °C, pH 5.0, 8 h, 30% ACN	10:90	10.2:89.8	-	Ajisaka et al., 1994

^a and ^b The donor/acceptor ratio was [donor] versus [acceptor] in mass and molar concentrations, respectively.

^c The transfer ratio was [sialyl-product] versus [Neu5Ac].

^d and ^e The total yield (%) was [sialyl-product] versus [donor] in molar and mass concentrations, respectively.

ACN, acetonitrile

DMSO, dimethylsulfoxide

Le^s, Lewis A (Gal β 1-3(Fuc α 1-4)GlcNAc)

Le^x, Lewis X (Gal β 1-4(Fuc α 1-3)GlcNAc)

T-antigen, T-(Thomsen-Friedenreich) antigen (Gal β 1-3GalNAc α 1-O-Ser/Thr)

Tn-antigen, Tn-(Thomsen-nouveau) antigen (GalNAc α 1-O-Ser/Thr)

structures used in *S. typhimurium* sialidase-catalysed reactions (Schmidt et al., 2000). (3) Reaction conditions, including acceptor concentration, temperature, pH and reaction time. A five-time increase of the concentration of the acceptor β Gal-OCH₃ resulted in a 5.6-time increase in the regioisomeric ratio (α -2,6: α -2,3) for *V. cholerae* sialidase (Thiem and Sauerbrei, 1991). The regioselectivity was also favoured at higher temperature and prolonged reaction time in either *V. cholerae* or *C. perfringens* sialidase-catalysed reaction (Schmidt et al., 2000). Such influences could be correlated to product build-up and subsequent degradation. Similarly, the ratio of (α -2,6: α -2,3) in the *B. fragilis* sialidase-catalysed trans-sialylation decreased 11.6-fold by extending reaction time from 3 h to 20 h, which was due to accumulation of 3'-SL and degradation of 6'-SL (Tanaka et al., 1995). Moreover, using the promiscuous trans-sialidase activity of a *P. multocida* SiaT (PmST), the reaction could be driven to either α -2,3- or α -2,6-trans-sialylation under combinatorial control of pH and reaction time (Guo et al., 2014/chapter 2).

1.4 BACTERIAL SIALYLTRANSFERASE

Sialyltransferases (EC 2.4.99.-) catalyse the transfer of a sialic acid from an activated sugar nucleotide donor CMP-sialic acid to a variety of acceptor molecules, usually at the 3- or 6-position of a terminal Gal or *N*-acetylgalactosamine (GalNAc) residue, or the 8- or 9-position of a terminal sialic acid residue. Bacteria expressing SiaTs are usually pathogens that display sialooligosaccharides structurally identical to mammalian glycolipids on their cell surface in order to evade the host's immune response (Audry et al., 2011; Li and Chen, 2012; Moran et al., 1996; Schwardt et al., 2006).

1.4.1 Structural and biochemical properties

Based on sequence homology, all bacterial SiaTs reported so far have been classified into four glycosyltransferase (GT) families (GT 38, 42, 52 and 80) in the CAZY database (Coutinho et al., 2003; Yamamoto et al., 2008). GT38 contains exclusively bacterial polySiaTs, while GT 42, GT52 and GT 80 contain bacterial lipooligosaccharide (LOS) SiaTs (Table 1.4).

The tertiary structures of bacterial SiaTs characterized so far fall into GT-A and GT-B folds. *C. jejuni* α -2,3/2,8-SiaT (Cst-II) and α -2,3-SiaT (Cst-I) both belonging to GT42 adopt a modified GT-A structure, *i.e.* a single $\alpha/\beta/\alpha$ sandwich consisting of an N-terminal Rossmann nucleotide-binding domain and a C-terminal lid-domain that encompasses the active site region (Fig. 1.5A) (Chiu et al., 2004 and 2007). On the other hand, *P. multocida* α -2,3/2,6-SiaT (Δ 24PmST1), *Vibrionaceae*

Photobacterium sp. α -2,6-SiaT (Δ 16psp26ST) and *Photobacterium phosphoreum* lipooligosaccharides α -2,3/2,6-SiaT (Δ Npp23ST) all belonging to GT80 as well as *Neisseria meningitidis* α -2,3/2,6-SiaT (NST) belonging to GT52 display a modified GT-B structure consisting of two separate Rossmann domains with a deep nucleotide-binding cleft located between the two domains (Fig. 1.5B) (Iwatani et al., 2009; Kakuta et al., 2008; Lin et al., 2011; Ni et al., 2007).

Table 1.4 Relationship between enzyme activities and bacteria. Regiospecificity for SiaT reaction is listed as well as reported side trans-sialidase (TS)/sialidase (SA) activity.

GT family	Origin	Enzyme activities		Reference
		SiaT activity	TS/SA activity	
38	<i>Escherichia coli</i> K1	α -2,8-SiaT	-	Cho and Troy, 1994; Steenbergen et al., 1992
	<i>Escherichia coli</i> K92	α -2,8/2,9-SiaT		Shen et al., 1999
	<i>Neisseria meningitidis</i> group B	α -2,8-SiaT		Edwards et al., 1994; Freiburger et al., 2007
42	<i>Neisseria meningitidis</i> group C	α -2,9-SiaT		Peterson et al., 2011
	<i>Campylobacter jejuni</i>	α -2,3/2,8-SiaT (Cst-II ^a)	α -2,8-TS; α -2,8-SA	Chiu et al., 2004; Cheng et al., 2008
		α -2,3-SiaT (Cst-I ^a)	-	Chiu et al., 2007
	<i>Haemophilus influenzae</i>	α -2,3/2,8-SiaT (lic3B)		Fox et al., 2006
52	<i>Pasteurella multocida</i>	α -2,3-SiaT (PmST3)		Thon et al., 2012
	<i>Haemophilus influenzae</i>	α -2,3-SiaT (lic3A)	-	Hood et al., 2001
	<i>Neisseria gonorrhoeae</i>	α -2,3-SiaT		Gilbert et al., 1996
	<i>Neisseria meningitidis</i>	α -2,3-SiaT		Gilbert et al., 1997
		α -2,3/2,6-SiaT ^a		Wakarchuk et al., 2001
80	<i>Pasteurella multocida</i>	α -2,3-SiaT (PmST2)		Lin et al., 2011
	<i>Haemophilus ducreyi</i>	α -2,3-SiaT	-	Thon et al., 2011
	<i>Pasteurella multocida</i>	α -2,3/2,6-SiaT (Δ 24PmST1 ^a ; PmST)	α -2,3/2,6-TS; α -2,3/2,6-SA	Li et al., 2007
				Guo et al., 2014 and chapter 3
				Ni et al., 2007; Yu et al., 2005
	<i>Photobacterium damsela</i>	α -2,6-SiaT	α -2,6-TS; α -2,6-SA	Cheng et al., 2010; Yamamoto et al., 1996 and 1998b
	<i>Photobacterium leiognathi</i>	α -2,6-SiaT	α -2,6-SA	Mine et al., 2010b
	<i>Photobacterium leiognathi</i>	α -2,6-SiaT	-	Yamamoto et al., 2007
	<i>Photobacterium phosphoreum</i>	α -2,3-SiaT ^a	α -2,3-SA	Tsukamoto et al., 2007; Iwatani et al., 2009
	<i>Photobacterium</i> sp.	α -2,3-SiaT	α -2,3-SA	Tsukamoto et al., 2008;
		α -2,6-SiaT ^a	-	Kakuta et al., 2008
	<i>Vibrio</i> sp.	α -2,3-SiaT		Takakura et al., 2007

^a Enzyme structures have been solved.

Despite sequence and structure differences among bacterial SiaTs, the enzymes are inverting GTs that catalyse the formation of α -sialyl linkage from β -linked sialic acid in CMP-sialic acid donor substrate through a direct displacement mechanism (Fig. 1.6). A catalytic residue functions as a

general base that deprotonates the hydroxyl group of the acceptor to activate it as a nucleophile for attack of anomeric center of the sialic acid moiety in the donor. Through a single oxocarbenium ion-like transition state, SiaTs catalyse the synthesis of sialyl linkage between acceptor and donor substrates with inversion of the configuration at C2. In SiaTs from GT42, a His residue (H202 in Cst-I and H188 in Cst-II) is a potential general base, while an Asp residue in SiaTs from GT80 (D141 in $\Delta 24\text{PmST1}$), and from GT52 (D528 in NST), serves as a general base. A second catalytic residue serves as a general acid to protonate the oxygen on the phosphate of the nucleotide sugar and thereby facilitates the departure of the CMP leaving group. In Cst-I and Cst-II proteins, two conserved tyrosine residues assist the departure of the phosphate group, while in case of SiaTs from GT80 and GT52 a His residue is a general acid to stabilize the CMP leaving group (Li and Chen, 2012; Iwatani et al., 2009; Kakuta et al., 2008; Lin et al., 2011; Ni et al., 2007). Two conserved motifs (D/E-D/E-G and H-P) are present throughout SiaTs from GT38, GT52 and GT80, and in SiaTs from GT80 and GT52 the H-P motif contains the catalytic acid (His). The motif D/E-D/E-G contains the catalytic base (Asp) in SiaTs (GT80), however the conserved motif is not located within the active site of NST (GT52) (Freiberger et al., 2007; Lin et al., 2011; Yamamoto et al., 2008).

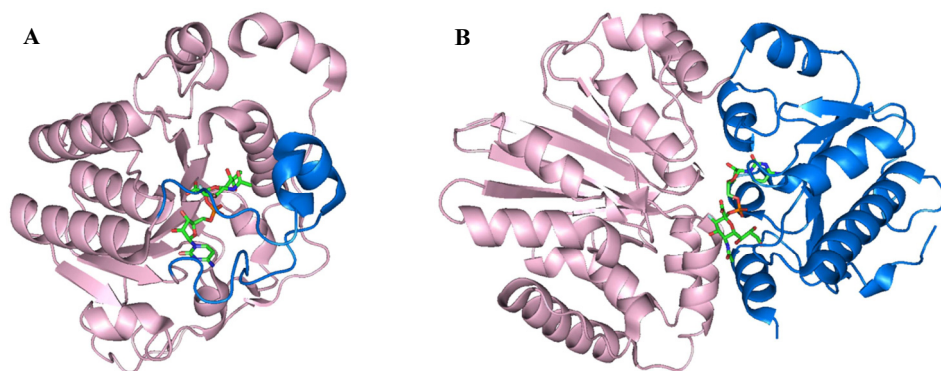


Fig. 1.5 Structures of *Campylobacter jejuni* Cst-II (PDB code 1RO7) and *Pasteurella multocida* $\Delta 24\text{PmST1}$ (PDB code 2IHK) in complex with CMP-3F-Neu5Ac. (A) Cst-II adopts a modified GT-A fold; (B) $\Delta 24\text{PmST1}$ adopts a modified GT-B fold. For both structures, N-terminal and C-terminal domains are colored in light pink and marine, respectively, and the ligand CMP-3F-Neu5Ac is shown in green-colored sticks.

The monomeric molecular weights of bacterial SiaTs generally lie between 40-60 kDa. Most enzymes are monomers, but there are exceptions, such as *N. meningitidis* α -2,3-SiaT (dimer), and

Cst-I and Cst-II (tetramer) (Chiu et al., 2004 and 2007; Gilbert et al., 1997). Bacterial SiaTs are usually membrane-associated proteins. In particular, α -2,3-SiaTs from *N. meningitidis* and *N. gonorrhoeae* have been verified to be surface-exposed outer-membrane proteins (Gilbert et al., 1996; Shell et al., 2002). SiaTs from marine bacteria *Photobacterium* sp. are predicted to be lipoproteins that are translocated across the cytoplasmic membrane to the periplasma (Takakura et al., 2007; Tsukamoto et al., 2007 and 2008; Yamamoto et al., 1998b and 2007). Bacterial SiaTs are metal-independent GTs. In some cases the SiaT activity can be stimulated by divalent ions (Gilbert et al., 1996 and 1997; Peterson et al., 2011; Willis et al., 2008), however for *P. damsela* α -2,6-SiaT, Δ 24PmST1 and *Haemophilus ducreyi* α -2,3-SiaT, the activity is inhibited by increasing the concentration of Mn^{2+} (Li et al., 2007; Sun et al., 2008; Yu et al., 2005). Moreover, SiaTs from marine bacteria are normally more active in a high concentration of NaCl (Mine et al., 2010b; Takakura et al., 2007; Tsukamoto et al., 2007 and 2008; Yamamoto et al., 2007). The kinetic properties of bacterial SiaTs can be affected by the assay conditions (Freiberger et al., 2007; Mandrell et al., 1993; Regan et al., 1999). In general, the apparent K_m values for CMP-Neu5Ac range between 0.02 mM and 1.4 mM, however, the lowest K_m for CMP-Neu5Ac reported so far is 5.3 μ M for *N. gonorrhoeae* SiaT (Mandrell et al., 1993). The optimum pH values fall between 5.0 and 8.0. Particularly, marine bacteria SiaTs display optimum pH values in the acidic range except *Photobacterium leiognathi* α -2,6-SiaT (optimum pH at 8.0) (Yamamoto et al., 2007), and the temperature optima for marine bacteria SiaTs are in the range of 25-35 $^{\circ}$ C.

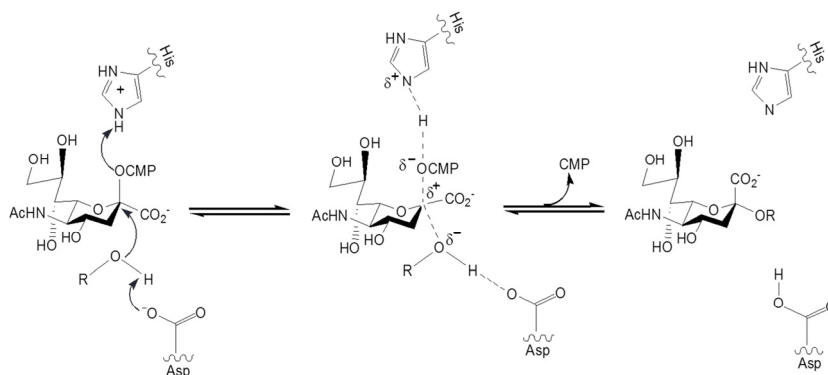


Fig. 1.6 Proposed catalytic mechanism for inverting SiaTs (GT80). R, glycoside.

A few bacterial SiaTs are bifunctional enzymes, capable of synthesizing two types of sialyl linkages, including Cst-II, *E. coli* α -2,8/2,9-polySiaT, *H. influenzae* α -2,3/2,8-SiaT, *N. meningitidis* α -2,3/2,6-SiaT, Δ 24PmST1 and PmST (Table 1.4). Some bacterial SiaTs have flexible donor substrate

specificity. For instance, Cst-II (Cheng et al., 2008), $\Delta 24\text{PmST1}$ (Yu et al., 2005), α -2,6-SiaTs from *P. damsela* (Kajihara et al., 1999; Yu et al., 2006b) and *Photobacterium* sp. JT-ISH-224 (Drouillard et al., 2010) are capable of using CMP-Neu5Ac, CMP-Neu5Gc, CMP-KDN or their derivatives (*e.g.* with sialic acid modifications at C-5 or C-9) as donor substrates for production of sialyloligosaccharides. Further, bacterial SiaTs have promiscuous acceptor substrate specificity. Marine bacteria SiaTs are less specific for the type of the second sugar from the non-reducing terminus and for the linkage between the terminal and second sugars as compared to the mammalian counterparts. For instance, α -2,3-SiaTs from *P. phosphoreum*, *Photobacterium* sp. JT-ISH-224 and *Vibrio* sp. JT-FAJ-16 can catalyse the transfer of sialic acid to both α - and β -galactosides (Mine et al., 2010b; Takakura et al., 2007; Tsukamoto et al., 2007 and 2008; Yamamoto et al., 2007), and *P. damsela* α -2,6-SiaT acts on both fucosyl- and sialyl-trisaccharides (Yamamoto et al., 1996).

Several SiaTs have been shown to also catalyse trans-sialidase/sialidase activities, including Cst-II (Cheng et al., 2008), *Photobacterium damsela* α -2,6-SiaT ($\Delta 15\text{Pd2,6ST}$) (Cheng et al., 2010), $\Delta 24\text{PmST1}$ (Yu et al., 2005) and its homologue PmST (Guo et al., 2014). Generally, promiscuous activities share the main active site features with the native activity (Khersonsky and Tawfik, 2010). In case of $\Delta 24\text{PmST1}$ and PmST, it has been suggested that their α -2,3-sialidase as well as α -2,3-trans-sialidase activities follow a retaining mechanism, in which D141 is a possible general acid/base and His311 is a possible nucleophile (Guo et al., 2014; Sugiarto et al., 2011). In some cases, the same catalytic residue can act in a different protonation state in the native compared to the promiscuous function (Khersonsky and Tawfik, 2010). The different activities of $\Delta 24\text{PmST1}$ have different pH optima: the α -2,3-SiaT (pH 7.5-9.0), α -2,3-trans-sialidase (pH 5.5-6.5) and α -2,3-sialidase activities (pH 5.0-5.5), possibly being involved in modulating charges and consequently functionalities of critical residues (Sugiarto et al., 2011; Yu et al., 2005). Moreover, promiscuous activities are generally less efficient than the native activity, however the difference in $k_{\text{cat}}/K_{\text{m}}$ value falls in a broad range (1-9 orders of magnitude) (Khersonsky and Tawfik, 2010). In case of these SiaTs, the trans-sialidase and sialidase activities are 5-3150 folds less efficient as compared to the native function (Cheng et al., 2008 and 2010; Guo et al., 2014; Yu et al., 2005).

1.4.2 Bacterial SiaT-catalysed synthesis of sialyloligosaccharides

Compared to bacterial sialidases, bacterial SiaTs have the advantage of achieving high yields and formation of regiospecific products, but its application for larger-scale production of

sialyloligosaccharides can be hampered by prerequisite of the expensive donor substrate CMP-sialic acid (Desmet and Soetaert, 2011; Schmidt et al., 2000). This drawback might be overcome by use of an *in situ* CMP-Neu5Ac regeneration system, which uses PEP, adenosine-5'-triphosphate (ATP) and CMP as synthetic substrates and nucleoside monophosphate kinase (NMK), pyruvate kinase (PK), CMP-Neu5Ac synthetase and pyrophosphatase (PPase) as catalysts (Ichikawa et al., 1991b). Mimicking the natural way for synthesis of sialyloligosaccharides, a one-pot multi-enzyme enzymatic approach has been developed with or without *in situ* cofactor regeneration system. A typical one-pot multi-enzyme sialylation starts from sialic acid aldolase-catalysed condensation reaction that converts sialic acid precursors, *i.e.* ManNAc, mannose (Man) or C-2/C-6 modified derivatives to sialic acids or C-5/C-9 modified derivatives. These compounds are subsequently activated by a CMP-sialic acid synthetase to form CMP-sialic acid, which is then used by a suitable SiaT to catalyse the formation of sialyloligosaccharides (Ichikawa et al., 1991a; Yu et al., 2006b). Moreover, taking the advantage of flexible donor- and acceptor-substrate specificity of bacterial SiaTs, *e.g.* $\Delta 24\text{PmST1}$ (Yu et al., 2005), $\Delta 15\text{Pd2,6ST}$ (Yu et al., 2006a) and Cst-II (Yu et al., 2009), Chen group has expanded the usage of one-pot multi-enzyme system for production of a large number of complex sialyloligosaccharides, including those with natural and non-natural sialic acid forms, different (α -2,3, α -2,6 or α -2,8) sialyl linkages and different underlying glycans. Isolation of intermediates is not required in the one-pot reaction, and typical yields for preparative-scale (>20 mg) synthesis are between 60% and 90%.

In addition, the use of SiaTs in bacterial cell factory allowed production of sialyloligosaccharides (examples are given in section 1.7).

1.5 CASEIN GLYCOMACROPEPTIDE (cGMP)

For industrial scale production of HMOs, it is paramount to identify a suitable, abundant and cheap sialyl donor. cGMP, the waste product from cheese manufacturing is a particular interesting candidate. It is the hydrophilic C-terminal fragment of bovine κ -casein (κ -CN), generated by chymosin cleavage of κ -CN between Phe105 and Met106. The soluble cGMP is released in the whey fraction, whereas the remaining part para- κ -CN precipitates into the cheese curd (Delfour et al., 1965). cGMP, containing 64 amino acids (residues 106-169 of κ -CN) (Fig. 1.7), is one of the most abundant proteins in whey with a concentration of 20-25% (Thomä-Worringer et al., 2006). Moreover, cGMP is unique in its amino acid composition as it is devoid of aromatic amino acids,

low in basic, acidic, and hydroxyl amino acids but rich in branched chain amino acids (Farrell et al., 2004). It is an acidic peptide with pI between 4 and 5.

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101 .....-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-
                                     Thr (Var. A)
121 Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro- -Thr-Glu-Ala-Val-Glu-
                                     Ile (Var. B)
                                     Asp (Var. A)
141 Ser-Thr-Val-Ala- -Leu-Glu-Asp-Ser-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-
                                     Ala (Var. B)

161 Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val

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Fig. 1.7 Amino acid sequence of cGMP with variants A (T126 and D148) and B (I126 and A148). Figure is adapted from Thomä-Worringer et al., 2006. Var. represents variant.

cGMP is a heterogeneous group of polypeptides mainly due to its genetic variance and post-translational modifications (*O*-glycosylation and phosphorylation). Originating from the precursor bovine κ -CN, two genetic variants (A and B) dominates in cGMP where two substitutions, *i.e.* Thr or Ile at position 126, and Asp or Ala at position 148, occur respectively (Fig. 1.7). All *O*-glycosylation and phosphorylation sites are exclusively situated in the cGMP region of κ -CN (Farrell et al., 2004; Mercier et al., 1973). Five mucin-types of carbohydrate moieties composed of Neu5Ac, Gal and GalNAc, have been identified in cGMP: monosaccharide GalNAc-*O*-R (0.8%), disaccharide Gal β 1-3GalNAc-*O*-R (6.3%), trisaccharide Neu5Ac α 2-3Gal β 1-3GalNAc-*O*-R (18.4%), trisaccharide Gal β 1-3(Neu5Ac α 2-6)GalNAc-*O*-R (18.5%), and tetrasaccharide Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)GalNAc-*O*-R (56%) (Saito and Itoh, 1992). Glycosylation of cGMP differs in the numbers of glycosylation sites. The glycans may be attached to six (in variant A) or five (in variant B) different Thr residues (Pisano et al., 1994) and probably also to one Ser residue (Kanamori et al, 1981; Zevaco and Ribadeau-Dumas, 1984). Fourteen glycosylated forms in addition to aglyco-forms have been identified in variant A (Mollé and Léonil, 1995).

Another source of heterogeneity is the variable level of phosphorylation, ranging from 1 to 3 phosphate groups identified per molecule (Mercier, 1981; Mollé and Léonil, 1995; Vreeman et al., 1986). Under certain conditions of pH and ionic strength, cGMP also displays heterogeneity in apparent molecular mass, which is much higher at neutral pH (20-50 kDa) than its theoretical value (7-9 kDa). This phenomenon is attributed to polymerization occurring at pH>4.5 and depolymerization occurring at more acidic pH ranges (Kawasaki et al., 1993; Morr and Seo, 1988;

Tanimoto et al., 1990). In contrast, Minkiewicz et al. (1996) suggested that the presence of hydrophilic and ionizable carbohydrate moieties leads to internal electrostatic and steric repulsion, thereby enlarging the voluminosity of cGMP.

cGMP shows a strong absorption at 205-217 nm but not at 280 nm due to the absence of aromatic amino acids. Therefore, differences in UV absorption at 210/280 nm are frequently used to characterize cGMP (El-Salam et al., 1996). The chromatography based methods for characterization of cGMP include size exclusion chromatography (Kawakami et al., 1992; Morr and Seo, 1988; Vreeman et al., 1986), ion exchange chromatography (Daultani et al., 2003; Léonil and Mollé, 1991; Nakano and Ozimek, 1999), reversed-phase chromatography (Coolbear et al., 1996; López-Fandiño et al., 1993) and hydrophobic interaction chromatography (Nakano and Ozimek, 2000), associated with NMR, MS and fourier transformed infrared spectroscopy (FTIR) techniques (Burgardt et al., 2014; Minkiewicz et al., 1996; Mollé and Léonil, 1995; Smith et al., 2002). Ion exchange chromatography is widely used for preparative-scale separation of cGMP from whey (Burton et al., 1987; Kawasaki and Dosako 1992; Saito et al., 1991), coupled with membrane filtration (Holst and Chartterton, 1999; Kawasaki et al., 1993; Kreuß et al., 2008). In particular, the pH-dependent molecular mass of cGMP can be used to influence its permeation behavior during the membrane filtration process.

1.6 BIOCATALYTIC PRODUCTION OF HMOs

Elephant milk oligosaccharides are the most structurally similar to HMOs (Kunz et al., 1999), however, large-scale use of these oligosaccharides is unfeasible. By use of biocatalytic synthesis approaches, HMO tri- and tetrasaccharides have been produced in kg quantities, however, the use of HMOs as bioactive substances in infant food, pharmaceuticals or for clinical trials is still limited by the ability to synthesize more complex structures in sufficient quantity and purity (Bode, 2012; Kunz et al., 2000). In general, two approaches are employed for biocatalytic production of HMOs, comprising *in vivo* production using metabolically engineered microorganisms (usually *E. coli* cells) and *in vitro* production using single or multiple enzymatic catalysis.

1.6.1 Production of lacto-*N*-oligosaccharides

In vivo production of lacto-*N*-oligosaccharides was illustrated by the study of Priem et al. (2002). A β -galactosidase-deficient *E. coli* strain was engineered by overexpressing one or two GT genes. Lactose added in the medium was internalized by β -galactoside permease and then glycosylated.

Using β -1,3-GlcNAcT gene resulted in production of 6 g/L lacto-*N*-trisaccharide II (GlcNAc β 1-3Gal β 1-4Glc), while using β -1,3-GlcNAcT and β -1,4-GalT genes, LNnT and lacto-*N*-neohexaose (LNnH, Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc) were produced with a yield of >5 g/L.

Enzymatic synthesis of LNT and LNnT involved β -1,3-GlcNAcT and β -galactoside as catalysts (Murata et al., 1999a; Miyazaki et al., 2010). The former enzyme from bovine serum catalysed the transfer of GlcNAc from UDP-GlcNAc to lactose for formation of lacto-*N*-trisaccharide II, which was then converted to LNT or LNnT using the donor pNP-Gal and *Bacillus circulans* β -1,3- or β -1,4-galactosidase, respectively. An alternative approach for LNT synthesis employed *Aureobacterium* sp lacto-*N*-biosidase-catalysed transglycosylation with pNP-Gal β 1-3GlcNAc as donor and lactose as acceptor (Murata et al., 1999a).

1.6.2 Production of SL and more complex sialylated HMOs

Endo et al. (2000) established a coupled-cell method using permeabilized cells (surfactant-treated) of a *Corynebacterium ammoniagenes* strain and three engineered *E. coli* strains. The *C. ammoniagenes* strain was responsible for synthesis of uridine-5'-triphosphate (UTP) from orotic acid, and the three *E. coli* strains overexpressed the genes of cytidine-5'-triphosphate (CTP) synthetase, CMP-Neu5Ac synthetase and *N. gonorrhoeae* α -2,3-SiaT respectively. This allowed production of 36 g/L 3'-SL from orotic acid, Neu5Ac and lactose. A single-cell method was established using a metabolically engineered *E. coli* strain (β -galactosidase-deficient) that was modified by inactivation of the gene encoding sialic acid aldolase and addition of CMP-Neu5Ac synthetase and *N. meningitidis* α -2,3-SiaT genes (Priem et al., 2002). This allowed production of 2.6 g/L 3'-SL from lactose and Neu5Ac that were internalized by β -galactoside permease and sialic acid permease, respectively. To avoid the use of expensive Neu5Ac, a pathway for *de novo* synthesis of Neu5Ac from UDP-GlcNAc was introduced in *E. coli* K12 by co-expressing the genes encoding UDP-GlcNAc-2-epimerase and SAS and disrupting ManNAc kinase and Neu5Ac aldolase genes. Using CMP-Neu5Ac synthetase and *N. meningitidis* α -2,3-SiaT genes, 25.5 g/L 3'-SL was produced with continuous supply of lactose (Fierfort and Samain, 2008). Similarly, using *Photobacterium* sp. α -2,6-SiaT gene led to production of 34 g/L 6'-SL (Drouillard et al., 2010). The whole-cell-based approach takes advantage of the metabolic machinery of the microorganisms, but one of the limitations is that acceptor substrates are restricted to those molecules that can be transported into the cells or synthesised *in vivo*.

Providing CMP-Neu5Ac and lactose as substrates, application of *Photobacterium* sp. JT-ISH-224 α -2,3-SiaT (Mine et al., 2010a) or *P. damsela* JT060 α -2,6-SiaT (Yamamoto et al., 1998a) led to *in vitro* production of 3'- or 6'-SL with a yield of >60%. Multiple enzymatic production of SL usually uses the one-pot multiple-enzyme system (mentioned in section 1.5.2), e.g., 3'-SL was produced from Neu5Ac and lactose in a yield of 79% using sialic acid aldolase, CMP-Neu5Ac synthetase and Δ 24PmST1 as catalysts (Yu et al., 2005). An interesting method was established for *in situ* generation of CMP-Neu5Ac from Neu5Ac and CTP using a fusion enzyme with CMP-Neu5Ac synthetase and *N. meningitidis* α -2,3-SiaT activity. Providing lactose, Neu5Ac, PEP, ATP and CMP, 67.8 g 3'-SL was produced in a 100 g scale reaction after 6 days (Gilbert et al., 1998).

Examples concerning bacterial sialidase-catalysed production of SL are given in Table 1.3, mostly using irreversible sialyl donor pNP-Neu5Ac. Application of TcTS allowed production of 3'-SL in a yield of 87% from pNP-Neu5Ac (Singh et al., 2000) and 75% from fetuin (Lee et al., 2002), respectively. For preparative-scale production of SL, the cheap donor substrate cGMP from dairy side-stream is preferable, e.g., the use of *A. ureafaciens* sialidase, *B. infantis* sialidase, or TcTS contribute to production of 0.2-0.8 mg/L SL, respectively (Mcjarrow et al., 2003; Pelletier et al., 2004). To maximize the yields, reaction conditions such as pH, temperature, time and ratio of donor versus acceptor concentration should be identified to favor trans-sialylation over hydrolysis reaction (Desmet and Soetaert, 2011). Trans-sialylation catalysed by TcTS (Holck et al., 2014) or two *Trypanosoma rangeli* sialidase (TrSA) mutants (Jers et al., 2014; Michalak et al., 2014) under optimised reaction conditions led to production of >1.5 g/L 3'-SL. In addition, using the additional α -2,3/2,6-trans-sialidase activity of PmST led to production of 1.8 g/L 3'-SL and 2.1 g/L 6'-SL at two differently optimised conditions (Guo et al., 2014). With the attempt to lower hydrolysis and enzyme cost as well as to enhance product yield, two studies using membrane reactor for continuous SL production were conducted, where *Streptococcus* sp. sialidase (Masuda et al., 2000) or a mutant enzyme derived from TrSA, namely TrSA₆ (TrSA with 6 mutations) (Zeuner et al., 2014) was used as catalyst. The former enzyme gave a total SL (3'- and 6'-SLs) yield of 2.03% from colominic acid in a 160-h continuous reaction, while the latter gave a 3'-SL yield of 74% from cGMP after seven consecutive runs (7×60 min).

With cGMP as donor substrate, 3'-sialyl-LNT (LST a) was produced from LNT using TcTS (Holck et al., 2014) or TrSA₆ (Michalak et al., 2014). TcTS was also used for catalysing α -2,3-sialylation of LNT from the donor 3'-SL (Johnson, 1999), and TrSA₆ was used for catalysing α -2,3-sialylation

of LNT, LNnT, lacto-*N*-fucopentaose I (LNFP I, Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc) and LNFP V (Gal β 1-3GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc) (Michalak et al., 2014).

1.6.3 Production of FL and more complex fucosylated HMOs

Koizumi et al. (2000) established a coupled-cell approach using a *C. ammoniagenes* strain responsible for formation of guanosine-5'-triphosphate (GTP) from guanosine-5'-monophosphate (GMP) and recombinant *E. coli* strains overexpressing guanosine-5'-diphosphate (GDP)-Fuc biosynthetic genes and *Helicobacter pylori* α -1,3-FUT gene. Providing GMP, Man and LNAc, an HMO intermediate Lewis X (Gal β 1-4(Fuc α 1-3)GlcNAc) was produced with a yield of 21 g/L in a 30-h reaction. A method for *in vivo* production of fucosylated HMOs (Drouillard et al., 2006; Dumon et al., 2001 and 2004) was established based on that used for *in vivo* production of LNnT (Priem et al., 2002). In this system, the *E. coli* strain was further engineered to provide GDP-Fuc by inactivating a gene involved in colanic acid synthesis and overexpressing a gene encoding a positive regulator of the colanic acid operon. Using *N. meningitidis* β -1,3-GlcNAcT and β -1,4-GalT genes and *H. pylori* FUT gene led to production of 0.5 g/L 3-FL, 1.7 g/L lacto-*N*-neofucopentaose I (LNFP I, Fuc α 1-2Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc), 3 g/L LNnFP V (Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc) or 1.7 g/L lacto-*N*-neodifucohexaose II (LNnDFH II, Gal β 1-4(Fuc α 1-3)GlcNAc1-3Gal β 1-4(Fuc α 1-3)Glc) from exogenous lactose. Further, the concentration of 3-FL was increased to 14 g/L with expressing only FUT gene and addition of more lactose (Drouillard et al., 2006). A different method using a metabolically engineered *E. coli* strain was patented (Huefner et al., 2010). A Fuc-salvage pathway for converting Fuc to GDP-Fuc was introduced in the β -galactosidase-deficient *E. coli* strain by adding the genes encoding *B. fragilis* Fuc kinase and Fuc-1-P-guanyltransferase and deleting the fucose-1-phosphate aldolase gene. Using either α -1,2- or α -1,3-FUT gene from *H. pylori* led to 2'- or 3-FL production, respectively.

With LNT or LNFP I as acceptor substrate, several human FUTs were able to catalyse trans-fucosylation for production of LNFP I or lacto-*N*-difucohexaose I (LNDFH I, Fuc α 1-2Gal β 1-3(Fuc α 1-4)GlcNAc β 1-3Gal β 1-4Glc), respectively (Nimtz et al., 1998; Miyazaki et al., 2010). Albermann et al. (2001) reported a method for multiple enzymatic synthesis of 2'-FL that involved three enzymes, *i.e.*, GDP-*D*-Man-4,6-dehydratase and GDP-4-keto-6-deoxy-*D*-Man-3,5-epimerase-4-reductase were responsible for converting GDP-Man to GDP-Fuc, which was subsequently used by *H. pylori* α -1,2-FUT for production of 2'-FL with a yield of 65%. Studies employing fucosidase for production of FL are limited. An example was given by Murata et al. (1999b) where application

of *Alcaligenes* sp. fucosidase contributed to production of 3'-FL (Fuc α 1-3Gal β 1-4Glc), giving a yield of 34%. This is however not the isomer found in human milk.

1.7 GLYCOSIDASE ENGINEERING FOR IMPROVED GLYCOSIDE SYNTHESIS

Improvement of transglycosylation efficiency of natural glycosidase can be achieved by protein engineering. The strategies for engineering glycosidases generally consist of rational design on the basis of available structural information as well as directed evolution with use of powerful screening approaches (Hancock et al., 2006). Using site-directed mutagenesis, the first strategy has been applied for *Bifidobacterium adolescentis* α -galactosidase (H497M) (Hinz et al., 2005), *Rhodothermus marinus* β -1,3-glucanase (M133C) (Neustroev et al., 2006), *Mucor hiemalis* endo- β -N-acetylglucosaminidase (Y271F) (Umekawa et al., 2008), *Serratia marcescens* chitinase (D313N/F396W) (Zakariassen et al., 2011), *Thermotoga neapolitana* β -glucosidase (N220F) (Lundemo et al., 2013), et al. These mutations play a role in either disfavoring the positioning of hydrolytic water molecule or favoring binding of incoming carbohydrate molecule in the aglycon subsite, thereby increasing transglycosylation activity. A noteworthy example is conferring trans-sialidase activity to the strict α -2,3-sialidase TrSA through five mutations (M96V/A98P/S120Y/G249Y/Q284P) (Paris et al., 2005). The five mutations (giving a mutant enzyme TrSA₅), selected by comparison to TcTS, defined a trans-sialidase-like binding site for the acceptor and modulated the sialic acid position, thereby contributing to ~1% trans-sialidase activity of that of TcTS. An additional single mutation I37L (giving TrSA₆) or G342A further increased the activity by 10-fold. Jers et al. (2014) reported that replacing a seven-amino-acid-motif on the edge of the acceptor binding cleft of TrSA₆ by that of TcTS (giving TrSA₁₃, TrSA with 13 mutations) reduced hydrolysis to promote trans-sialidase activity. They hypothesized that the reduced hydrolysis was due to disruption of the water network. Using free-energy computations, Pierdominici-Sottile et al. (2014) proposed five additional mutations based on TrSA₅ for enhancing the transfer activity, but this mutant was not tested experimentally.

Directed evolution, on the other hand, can be used for improving transglycosylation activity without prior knowledge of structure-function relationships. This strategy has been applied for enzymes such as *Thermus thermophilus* β -galactosidase (F401S/N282T; F401S/A205T/H365Y) (Feng et al., 2005; Koné et al., 2009), *Thermotoga maritima* α -1,2-fucosidase (T264A/Y267F/L322P) (Osanzo et al., 2007), *Neisseria polysaccharea* amylosucrase (V389L/N503I; R20C/F598S; N387D; E227G) (van der Veen et al., 2004 and 2006), *Thermoanaerobacterium thermosulfurigenes* cyclodextrin

glucanotransferase (S77P) (Kelly et al., 2008), and *Thermobacillus xylanilyticus* α -arabinofuranosidase (F26L/S319P/D471N) (Arab-Jaziri et al., 2013). Clearly, improvement of the transglycosylation/hydrolysis ratio is a prerequisite for obtaining mutants with good synthetic capability (Osanjo et al., 2007). Thus, the screening method typically contains two steps that enable rejection of mutants with high hydrolysis activity but keep those with increased transfer activity, taking advantage of the release of chromophoric or fluorophoric reaction products that are easily monitored. A good example was given by Koné et al. (2009) where a digital screening approach was used for engineering *T. thermophilus* β -galactosidase. Notably, some beneficial mutations are located in the second-shell around active site but can induce subtle conformational changes within the active site, while others are located further away (Feng et al., 2005; Kelly et al., 2008; Koné et al., 2009; Osanjo et al., 2007; van der Veen et al., 2004). The latter cases imply the difficulty of engineering glycosidases on a rational basis.

Glycosynthases represent a large group of retaining glycosidase mutants, in which the catalytic residue (Asp or Glu) is replaced by a non-nucleophile residue (typically Ala, Ser or Gly). The mutated enzymes lose the hydrolysis activity but can catalyse transglycosylation using an activated donor, such as a glycosyl fluoride with inverted anomeric configuration, in a quantitative yield (Bojarova and Kren, 2009; Williams and Withers, 2002). The first glycosynthase, derived from *Agrobacterium* sp. β -glucosidase (E358A), was able to catalyse the condensation of α -glucosyl fluoride with a range of carbohydrate alcohols in high yields (Mackenzie et al., 1998). The glycosynthase concept was firstly extended to an inverting glycosidase, *Bacillus halodurans* exo-xylanase. In this case saturation mutagenesis at the catalytic base D263 allowed generation of nine glycosynthase mutants, among which the mutant D263C gave the highest activity (Honda and Kitaoka, 2006). The application of glycosynthase technology could be expanded for HMO synthesis as manifested by a fucosynthase derived from *B. bifidum* α -1,2-fucosidase (Wada et al., 2008). The catalytic base mutant (D766G) was able to catalyse production of 2'-FL with a yield of 6% from a fucosyl fluoride. Using a similar approach led to creating a nucleophile mutant (Y370G) of *M. viridifaciens* sialidase, which acted as an inverting sialidase and catalysed the transfer of Neu5Ac from phenyl- β -Neu5Ac (Ph- β -Neu5Ac) to lactose giving a SL yield of ~13% (>90% of the product was the α -2,6-isomer) (Watson et al., 2006). However, the application of glycosynthase for large-scale production of oligosaccharides is restricted by the prerequisite of the unstable and hardly commercially available donors (*i.e.*, glycosyl fluorides and aryl glycosides).

1.8 HYPOTHESES AND OBJECTIVES

HMOs are structurally complex sugars that have a range of unique biological functions for human health. There is a potentially commercial use of HMOs for supplementation of infant formula. Large-scale production of HMOs decorated with α -2,3 bound sialic acid has been developed by use of several α -2,3-sialidases/trans-sialidases, especially TcTS. This PhD study was focused on biocatalytic production of sialylated HMOs, notably SL, containing both α -2,3 and α -2,6 sialyl linkages by use of other biocatalysts than TcTS, as well as improving catalytic proficiencies of the employed biocatalysts (*i.e.*, sialyltransferase and sialidase) by protein engineering.

The general hypotheses of this work were formulated as follows:

- *P. multocida* SiaT, displaying additional trans-sialidase activities, can be used for production of sialylated HMOs using cGMP as a sialyl donor.
- The regioselectivity of *P. multocida* SiaT can be altered towards either α -2,3- or α -2,6-trans-sialylation by optimization of reaction conditions and/or protein engineering.
- Protein engineering can be used to improve hydrolytic sialidase activity of *M. viridifaciens* sialidase that catalyses cleavage of α -2,3, α -2,6 and α -2,8 sialyl linkages.

The main objectives of this work were:

- To identify optimum reaction conditions for *P. multocida* SiaT-catalysed production of 3'- and 6'-SLs.
- To modulate the regioselectivity of *P. multocida* SiaT via site-directed mutagenesis for effective production of 3'- or 6'-SL, respectively.
- To apply site-saturation mutagenesis for improving hydrolase activity of *M. viridifaciens* sialidase.

A previous study (Yu et al., 2005) has reported that a *P. multocida* α -2,3/2,6-SiaT has trans-sialidase activity. In paper I, a *P. multocida* SiaT (a homologue enzyme with three amino acid differences compared to the former one) displaying α -2,3- and α -2,6-trans-sialidase activities was examined, and the reaction conditions were investigated for maximal production of 3'- and 6'-SLs with use of cGMP and lactose as substrates. The later work (paper II) aimed to alter the regioselectivity of this SiaT notably towards α -2,6-trans-sialylation via site-directed mutagenesis. Based on analysis of crystal structures of an α -2,3 and an α -2,6 SiaT, a His residue (corresponding

to Pro residue in *P. multocida* SiaT) was suggested to determine α -2,6-regiospecificity in SiaTs (Kakuta et al., 2008). In this work, we introduced a single mutation P34H in the *P. multocida* SiaT and tested the mutant in both trans-sialylation and sialyltransferase reactions. In addition, another four mutations (E271F, R313Y, E271F/R313Y and M144D) that significantly decreased sialidase activity but maintained SiaT activity (Sugiarto et al., 2011 and 2012) were also introduced in the enzyme and the derived mutants were tested in trans-sialylation reactions.

M. viridifaciens sialidase exhibits hydrolase activity for cleavage of α -2,3, α -2,6 and α -2,8 sialyl linkages. The work (paper III) was focused on improving hydrolase activity of *M. viridifaciens* sialidase by protein engineering. 20 amino acids in or near the sialic acid binding site were targeted for site-saturation mutagenesis and mutant libraries were tested on a natural substrate (cGMP) and an artificial substrate (pNP-Neu5Ac), respectively. Using the PoPMuSiC software, the stability change $\Delta\Delta G$, a measure of potential chemical variation was analysed for each amino acid. The correlation between computed $\Delta\Delta G$ and activity of mutants gave an assessment of the evolvability of catalytic proficiency of the sialidase towards the artificial substrate and the natural one, respectively.

2. ENZYMATIC PRODUCTION OF 3'- AND 6'-SIALYL GLYCANS

This chapter is extended in the form of paper I and II.

2.1 Hypotheses and objectives

cGMP, the waste product from cheese manufacturing, is an abundant and cheap sialyl donor for large-scale production of sialylated HMOs. Biocatalytic synthesis of α -2,3-sialyl-HMOs and analogues thereof (e.g., SL, LST, and Sia-GOS) from cGMP have been catalyzed by TcTS (Holck et al., 2014; Pelletier et al., 2004; Sallomons et al., 2013), or TrSA mutants (Jers et al., 2014; Michalak et al., 2014). In this study, a *Pasteurella multocida* SiaT was found to possess promiscuous α -2,3- and α -2,6-trans-sialidase activities. We hypothesized that this enzyme can be used for production of both α -2,3- and α -2,6-sialyl glycans from cGMP. The objectives of this study were to identify optimal reaction conditions for efficient and regioselective synthesis of α -2,3- and α -2,6-sialyloligosaccharides as well as to modulate the regioselectivity of the enzyme towards either α -2,3- or α -2,6-trans-sialylation by protein engineering.

2.2 Experimental considerations

Paper I

We first observed that when using pNP-Neu5Ac or cGMP as donor and lactose as acceptor, PmST was able to catalyse synthesis of not only 3'-SL but also 6'-SL. 3'-SL was the dominant product while 6'-SL was formed in a much lower rate. We thereafter examined the possibilities of maximally producing these two compounds by PmST catalysis. Statistically designed experiments were conducted, considering lactose concentration (50-150 mM), pH (4.4-6.4) and temperature (30-50 °C) at different reaction times (up to 8 h).

During the PmST-catalyzed trans-sialylation reaction, we found that the initial product of 3'-SL was converted to 6'-SL, indicating a uniquely promiscuous trans-sialidase activity of PmST. The kinetic analysis of this activity was done by reacting the enzyme under the optimal condition (pH 5.4-40 °C) on different concentrations of 3'-SL (up to 400 mM) with lactose concentration being constant (100 mM).

The trans-sialylation ability of PmST was also examined with GOS served as acceptors. The synthesized Sia-GOS were treated by an α -2,3-sialidase and then analysed by MALDI-MS to demonstrate the presence of both α -2,3 and α -2,6 sialyl linkages in the products.

Paper II

Four mutants (E271F, R313Y, E271F/R313Y and M144D) derived from a PmST homologue enzyme possessed significantly decreased sialidase activity but maintained SiaT activity (Sugiarto et al., 2011 and 2012). These mutations were introduced to PmST to examine their effect on trans-sialidase activity. Another mutation P34H that potentially determines α -2,6-regiospecificity (Kakuta et al., 2008) was also introduced to PmST. We then evaluated trans-sialylation ability of the five PmST-derived mutants under the optimal condition (pH 5.4-40 °C, 5% cGMP and 100 mM lactose) as identified in the study of Paper I. With respect to the P34H mutant, its native α -2,6-SiaT activity as well as pH effect on its α -2,6-trans-sialidase activity were also examined.

Three mutants (E271F, R313Y and E271F/R313Y) exhibited α -2,3-trans-sialylation preference and the P34H mutant predominantly catalysed synthesis of α -2,6 sialyl linkage. In order to evaluate the impact of donor linkage on the product formation rate for each mutant, we isolated the individual activities, that is, use 3'-SL as donor to monitor 6'-SL production and vice versa, and use the alternative donor 3'-SLN and 6'-SLN to follow production of 3'-SL and 6'-SL respectively. The kinetic parameter for α -2,6-trans-sialidase activity (using 3'-SL as donor) of P34H was also estimated.

2.3 Main results and conclusion

pH was the only factor influencing PmST-catalysed 3'-SL production and the interaction effect for pH \times temperature was significant throughout the reaction. Curvature effects for both temperature and pH (*i.e.*, pH \times pH, temp \times temp interactions) were also observed. For shorter reaction times (0.5 h and 2 h), the optimum condition for 3'-SL production was pH 5.4 and 40 °C. After 4 h, the optimum shifted toward pH 6.4 and 50 °C.

For 6'-SL production, none of the individual factors showed significant effect on the yield except pH at 0.5 h. However, curvature effects for pH and temperature (*i.e.*, pH \times pH, temp \times temp) were significant. The interaction effect for pH \times temperature was significant at the initial stage (0.5 h and 2 h). The optimum condition for 6'-SL production was pH 5.4 and 40 °C throughout the reaction.

When reacting the enzyme at 40 °C using 5% cGMP and 100 mM lactose as substrates, the maximum yield of 3'-SL (2.75 mM) was achieved at pH 6.4 after 6 h, and the largest concentration of 6'-SL (3.33 mM) was achieved at pH 5.4 after 8 h. 6'-SL was formed from α -2,3 bound sialic acid in cGMP as well as from 3'-SL produced in the reaction. For favoring production of either SL isomer, it was key to terminate the reaction at an appropriate time.

The k_{cat}/K_m value for α -2,6-trans-sialidase activity (using 3'-SL as donor) of the enzyme at the optimized condition was 23.22 M⁻¹ s⁻¹. When GOS served as acceptors, the enzyme was able to catalyse synthesis of both 3'- and 6'-Sia-GOS.

The PmST_{M144D} mutant exhibited very low trans-sialidase activity. The mutant enzymes PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y} all preferentially catalysed synthesis of 3'- SL over 6'- SL. The best PmST_{E271F/R313Y} gave a maximal yield of 3'-SL (~4.5 mM) after 10 h, with <0.1 mM 6'-SL produced. When a total SL yield of ~50% was obtained from cGMP, the regioisomeric ratio (α -2,3: α -2,6) and the transfer ratio for PmST_{E271F/R313Y} were 128- and 5-folds higher than those for the wild type enzyme, respectively.

When using 3'-SLN as donor for 3'-SL production, PmST_{E271F} maintained its α -2,3-trans-sialidase activity, while PmST_{R313Y} and PmST_{E271F/R313Y} retained 77% and 67% of the wild-type-level activity. When using 3'-SL as donor, PmST_{E271F/R313Y} exhibited the lowest α -2,6-trans-sialidase activity. This mutant enzyme displayed a larger reduction in α -2,6- compared to α -2,3-trans-sialidase activity, effectively shifting the specificity towards α -2,3-trans-sialylation.

The PmST_{P34H} mutant displayed a distinct preference for 6'-SL synthesis during the entire course of reaction, however, the total SL yield was consistently and significantly lower than that of the wild type enzyme. Further, PmST_{P34H} acquired a substantially higher α -2,6-SiaT activity (980-fold) than the wild type enzyme, while this increase was accompanied by loss of α -2,3-SiaT activity. The ability of this mutant enzyme to use α -2,3 bound sialic acid as donor for α -2,3-trans-sialylation was almost abolished as well. However, using this donor for α -2,6-trans-sialylation gave a wild-type-level activity. The k_{cat}/K_m value for α -2,6-trans-sialidase activity (using 3'-SL as donor) of PmST_{P34H} at pH 5.4 and 40 °C was 31.2 M⁻¹ s⁻¹, similar to that of the wild type enzyme. Both the wild type enzyme and PmST_{P34H} were capable of catalysing the hydrolysis and transfer of α -2,6 bound sialic acid.



A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3'-sialyl and 6'-sialyl glycans

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ABSTRACT

This study examined a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities. The enzyme catalyzed trans-sialylation using either 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminic acid or casein glycomacropeptide (whey protein) as the sialyl donor and lactose as the acceptor, resulting in production of both 3'-sialyllactose and 6'-sialyllactose. This is the first study reporting α -2,6-trans-sialidase activity of this sialyltransferase (EC 2.4.99.1 and 2.4.99.4). A response surface design was used to evaluate the effects of three reaction parameters (pH, temperature, and lactose concentration) on enzymatic production of 3'- and 6'-sialyllactoses using 5% (w/v) casein glycomacropeptide (equivalent to 9 mM bound sialic acid) as the donor. The maximum yield of 3'-sialyllactose (2.75 ± 0.35 mM) was achieved at a reaction condition with pH 6.4, 40 °C, 100 mM lactose after 6 h; and the largest concentration of 6'-sialyllactose (3.33 ± 0.38 mM) was achieved under a condition with pH 5.4, 40 °C, 100 mM lactose after 8 h. 6'-sialyllactose was presumably formed from α -2,3 bound sialic acid in the casein glycomacropeptide as well as from 3'-sialyllactose produced in the reaction. The k_{cat}/K_m value for the enzyme using 3'-sialyllactose as the donor for 6'-sialyllactose synthesis at pH 5.4 and 40 °C was determined to be $23.22 \pm 0.7 \text{ M}^{-1} \text{ s}^{-1}$. Moreover, the enzyme was capable of catalyzing the synthesis of both 3'- and 6'-sialylated galactooligosaccharides, when galactooligosaccharides served as acceptors.

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1. Introduction

Human milk oligosaccharides (HMOs) are the third most abundant component (5–10 g/L) in human milk (Bode, 2009). About 180 different HMOs species have been identified recently in a pooled human milk sample from five individuals, and nearly 16% of the total oligosaccharide abundances were found correspond to sialylated oligosaccharides (Ninonuevo et al., 2006). The structural diversity and complexity confer unique biological functions on HMOs. Sialylated HMOs, consisting of N-acetylneuraminic acid (Neu5Ac) attached to galactose or N-acetylgalactosamine (GalNAc) through α -2,3 or α -2,6 linkage, are known to have both anti-infective and immuno-modulating effects (Bode, 2012). They are also believed to influence brain development and cognitive capacity in the newborn infant (Wang, 2009; Wang et al., 2003). The sialyllactose (3'- and 6'-sialyllactoses) content ranges from 0.4 to 0.8 g/L and the isomers of monosialylated and disialylated lacto-N-tetraose are present in lower amounts in human milk (Kunz et al., 2000). In contrast, only trace amounts of sialylated oligosaccharides

Abbreviations: ATP, adenosine-5'-triphosphate; cGMP, casein glycomacropeptide; CMP-NeuAc, cytidine-5'-monophospho-N-acetylneuraminic acid; CTP, cytidine-5'-triphosphate; CV, column volume; GalNAc, N-acetylgalactosamine; GOS, galactooligosaccharides; HMOs, human milk oligosaccharides; HPAEC-PAD, high-performance anion exchange chromatography; LacMu, 4-methylumbelliferyl β -D-lactoside; LC-MS, capillary liquid chromatography/mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; NDV, Newcastle disease virus; Neu5Ac, N-acetylneuraminic acid; N-His6-tag, N-terminal His₆-tag; PEP, phosphoenolpyruvate; PGC, porous graphitic carbon; PmST, *Pasteurella multocida* sialyltransferase; pNP-Neu5Ac, 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminic acid; RSM, response surface methodology; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sia-GOS, sialylated galactooligosaccharides; TLC, thin-layer chromatography.

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are present in bovine milk, and consequently in the derived infant formula. Therefore, synthesis of sialylated HMOs for supplementation of infant formula has been suggested (Bode, 2009; Espinosa et al., 2007).

A variety of methods based on microbial systems have been developed for synthesis of sialylated HMOs, starting with the synthesis of sialyllactose. Endo and coworkers established a coupled-microbial method using *Corynebacterium ammoniagenes* and three engineered *Escherichia coli* strains overexpressing the genes of cytidine-5'-triphosphate (CTP) synthetase, cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NeuAc) synthetase and *Neisseria gonorrhoeae* α -2,3-sialyltransferase (SiaT), respectively (Endo et al., 2000). This allowed production of 57 mM 3'-sialyllactose from a 12-h reaction using orotic acid, Neu5Ac and lactose. A single-cell method was established by engineering *E. coli* K12 that enabled production of 4.1 mM 3'-sialyllactose from lactose and Neu5Ac supplemented to the medium (Priem et al., 2002). This strain was further refined to allow *de novo* synthesis of Neu5Ac. Using either *Neisseria meningitidis* α -2,3-SiaT or *Photobacterium* sp. α -2,6-SiaT gene led to production of 40.3 mM 3'-sialyllactose (Fierfort and Samain, 2008) or 53.7 mM 6'-sialyllactose (Drouillard et al., 2010), respectively. Despite these promising results, the application of the cell-based systems to a wide range of diverse acceptor molecules might be hampered by the need for efficient uptake or *in vivo* production of the acceptor.

As an alternative, several enzymatic methods have been developed. An interesting approach involved a fusion protein having both CMP-Neu5Ac synthetase and α -2,3-SiaT activities, which was utilized to produce 3'-sialyllactose from lactose, Neu5Ac, phosphoenolpyruvate (PEP), adenosine-5'-triphosphate (ATP) and CMP (Gilbert et al., 1998). When providing CMP-Neu5Ac and lactose as substrates, a *Pasteurella multocida* α -2,3/2,8-SiaT was capable of synthesizing 3'-sialyllactose with a yield of 2.7 mM after a 16-h reaction (Endo and Koizumi, 2004). Besides the use of SiaTs, trans-sialidases/sialidases have also been employed. The *Bacteroides fragilis* sialidase catalyzed trans-sialylation from colominic acid to lactose, resulting in synthesis of both 3'- and 6'-sialyllactoses with a total yield of 0.14% (Tanaka et al., 1995). Using casein glycomacropeptide (cGMP) from dairy streams and lactose as substrates, sialyllactose was produced from either *Arthrobacter ureafaciens* or *Bifidobacterium infantis* sialidase-catalyzed reaction (McJarrow et al., 2003). As well, methods for enriching bovine milk with 3'-sialyllactose and 3'-sialylated galactooligosaccharides (3'-Sia-GOS) produced by the *Trypanosoma cruzi* α -2,3-trans-sialidase-catalyzed trans-sialylation were patented (Pelletier et al., 2004; Sallomons et al., 2013).

Previously, a *P. multocida* α -2,3/2,6-SiaT (tPm0188Ph) has been produced in *E. coli* by Yu et al. (2005). The recombinant tPm0188Ph was found to be a multifunctional enzyme, i.e., an α -2,3-SiaT that catalyzes the transfer of a sialic acid residue from CMP-Neu5Ac to galactosides and forms α -2,3 sialyl linkage; an α -2,6-SiaT that forms α -2,6 sialyl linkage much less efficiently; a sialidase that cleaves α -2,3 but not α -2,6 sialyl linkage; a trans-sialidase that transfers sialic acid from α -2,3-sialosides (not α -2,6-sialosides) to galactosides. In this study, a *P. multocida* sialyltransferase (PmST) gene (Genbank accession number AAK02272), which possesses three amino acid differences (N105D, Q135R, and E295G) compared to tPm0188Ph, was overexpressed in *E. coli*. The objective of the work was to examine the PmST-catalyzed trans-sialylation in order to preliminarily assess the efficacy of its trans-sialidase activities for producing sialosides. In a reaction mixture containing cGMP, lactose and recombinant PmST, we observed that 3'-sialyllactose but also 6'-sialyllactose were formed. Thereafter, using response surface methodology (RSM), we examined the effects of pH, temperature and lactose concentration on production of 3'- and 6'-sialyllactoses.

2. Materials and methods

2.1. Chemicals and reagents

Phusion DNA polymerase, T4 DNA ligase, *Nco*I, *Eco*311 and *Kpn*I were purchased from Thermo Scientific (Waltham, USA). Protein standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, USA). 85% orthophosphoric acid and 25% ammonium were obtained from VWR (Denmark). 3'-sialyllactose sodium salt and 6'-sialyllactose sodium salt were purchased from Carbosynth (UK). 2-O-(p-nitrophenyl)- α -D-N-acetylneuraminic acid (pNP-Neu5Ac, containing a trace amount of Neu5Ac) was provided by Department of Chemistry, DTU. cGMP was supplied by Arla Foods (Viby, Denmark). The sialic acid content was 0.18 ± 0.011 mmol per gram of cGMP. Before use impurities of low molecular weight in the cGMP solution were removed by filtration through a Ultracel regenerated cellulose membranes (5 kDa cutoff) (Merk Millipore, USA). GOS were purchased from Gulio Gross (Trezzano, Italy). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Strain and plasmid

A gene encoding a putative *P. multocida* SiaT lacking the residues 2–25 (Genbank accession number AAK02272) was synthesized by Geneart (Life technologies, Germany) (the nucleotide and amino acid sequences given in Fig. S1). *E. coli* DH5 α was used for the maintenance and manipulation of plasmids. The expression host *E. coli* BL21(DE3)plysS (Novagen, USA) and the expression vector pETM-11 (EMBL, Germany) were utilized for the expression of PmST.

2.3. DNA manipulation and strain construction

The gene encoding PmST was PCR amplified using primers 5'-ATCGGGTCTCCCATGAAAACAATCAGCGCTGTATCTGG and 5'-CGGGGTACCTTACAGCTGTTTCAGGCTATCCAA (restriction sites underlined). The PCR product was digested with *Eco*311 and *Kpn*I and inserted in pETM-11 between *Nco*I and *Kpn*I in frame with an N-terminal His₆-tag (N-His₆-tag). The resulting recombinant plasmid was used to transform *E. coli* BL21(DE3)plysS.

2.4. Expression and purification of PmST

E. coli BL21(DE3)plysS harboring the recombinant plasmid was cultured in auto-induction medium ZYM-5052 (Studier, 2005) for 24 h with shaking at 30 °C. The cell pellets were harvested by centrifugation (20 min at 5000 g) and re-suspended in binding buffer (20 mM citrate-phosphate buffer, 100 mM NaCl, 15 mM imidazole, pH 7.4). Cells were lysed by sonication and centrifuged at 5000 g for 20 min. The supernatant was subjected to sterile filtration through a 0.45 μ m filter and subsequently loaded onto a 5 mL Ni²⁺-sepharose HisTrap HP column (GE healthcare, UK). The purification was carried out using the method described by Silva et al., 2011. The fractions containing N-His₆-tagged PmST were collected and then desalted using PD-10 desalting columns (GE Healthcare) to remove imidazole. Protein concentrations were determined using the BCA protein assay (Thermo scientific) with BSA as the standard.

2.5. Response surface design of trans-sialylation reactions

A quadratic central composite face centered design was used to generate factor combinations and multiple linear regression models were fitted to the data using MODDE 7.0 (Umetrics AB, Sweden). The investigated factors were as follows: pH 4.4, 5.4 and 6.4; temperatures 30 °C, 40 °C and 50 °C; and lactose concentrations 50 mM, 100 mM and 150 mM. Each experimental design consisted of 17

runs with three center points and was conducted at 0.5 h, 2 h, 4 h, 6 h and 8 h, respectively. The cGMP concentration was fixed as 5% (w/v), equivalent to 9 mM bound sialic acid. Reactions were initiated by addition of 0.35 mg/mL PmST and terminated by heating at 90 °C for 10 min. After centrifugation (5 min at 20,000g), the supernatants were subjected to filtration through vivaspin 500 (5 kDa cutoff) (Sartorius AG, Germany) and the concentrations of 3'-sialyllactose, 6'-sialyllactose and sialic acid in the permeates were determined by HPAEC-PAD as described below. All reactions were performed in duplicate.

2.6. Kinetic study of *trans*-sialidase activity for 6'-sialyllactose synthesis

The α -2,6-*trans*-sialylation reaction was assayed at 40 °C in 50 mM citrate-phosphate buffer (pH 5.4) using 3'-sialyllactose as the donor and lactose as the acceptor. The reactions were done with various concentrations of 3'-sialyllactose (20 mM, 50 mM, 100 mM, 200 mM, 300 mM and 400 mM) and a fixed concentration of lactose (100 mM) and initiated by addition of 0.35 mg/mL PmST. The initial velocity of each reaction was measured within 1 h. All reactions were performed in duplicate. Because the measurements were carried out at substrate concentrations far below the K_m value, the k_{cat}/K_m value was derived from a linear fit $v = [E_0][S_0](k_{cat}/K_m)$.

2.7. Enzymatic production and purification of Sia-GOS

An 8-h reaction (10 mL) was carried out at 40 °C in 20 mM citrate-phosphate buffer (pH 5.4) using 5% cGMP as the donor and 150 mM GOS as the acceptor. The reaction was initiated by addition of 0.35 mg/mL PmST and terminated by heating at 90 °C for 10 min. After centrifugation (5 min at 20,000g), the supernatant was subjected to filtration through vivaspin 20 (10 kDa cutoff) and subsequently applied to a 5 mL HiTrap Sepharose FF column (GE Healthcare). The separation was done using an AKTA purifier at a flow rate of 5 mL/min while monitored at 214 nm. The column was equilibrated with 5 column volume (CV) of deionized water (A). After sample loading, the unbound compound was removed by washing with 3 CV of A, and the target product was eluted with a linear gradient from 0% to 8.5% of 1 M ammonium formate (B). The fractions containing potential Sia-GOS were collected and lyophilized repetitively to remove ammonium formate. The product structures were determined by MALDI-MS as described below.

2.8. Thin-layer chromatography (TLC)

The TLC method for determination of 3'- and 6'-sialyllactoses and sialic acid was modified based on Maru et al. (1992). 2 μ L of samples or standards comprising β -lactose, Neu5Ac, 3'-sialyllactose and 6'-sialyllactose were applied to a TLC silica gel 60 plate (Merck Millipore). The plate was developed with the solvent 1-propanol–water–25% ammonium solution (37.5:15:10) for 2 h. Afterwards, the plate was stained by immersion with diphenylamine–aniline–phosphoric acid as dyeing reagent (Anderson et al., 2000) followed by heating at 120 °C for 15 min.

2.9. High-performance anion exchange chromatography (HPAEC-PAD)

A Dionex ICS-3000 system consisting of DP-3000 gradient pump, ED-3000 electrochemical detector coupled to an AS-3000 autosampler (Dionex Corp., USA) was used for HPAEC-PAD analysis. Oligosaccharides were separated using a CarboPac™ PA100 analytical column (4 mm \times 250 mm) with CarboPac™ PA100 guarded column (4 mm \times 50 mm) (Dionex Corp.) at 30 °C. A three-eluent

system comprising deionized water (A), 500 mM NaOH (B), and 500 mM sodium acetate (C) was used. The separation program started with 40% of B and 14% of C at 0 min, followed by a linear increase of C to 50% for the next 12 min. A 5-min re-equilibration step was run with 40% of B and 14% of C. The flow rate was kept at 1 mL/min through the entire program. β -lactose, Neu5Ac, 6'-sialyllactose and 3'-sialyllactose were eluted at retention times of approximately 1.94 min, 3.18 min, 4.02 min and 4.3 min, respectively. Calibration was performed with a series of standard solutions (concentration range 0.01–1 mM) treated by heating at 90 °C for 10 min. The peak area was used for quantification.

2.10. Capillary liquid chromatography/mass spectrometry (LC–MS)

An Agilent 1100 LC/Agilent 6340 ion trap MS system was used for LC–MS analysis. Oligosaccharides were separated using a Hypercarb porous graphitic carbon (PGC) column (0.32 mm \times 150 mm, 5 μ m) (Thermo scientific) at 30 °C. Samples in 10 mM ammonium bicarbonate were loaded onto the column. Gradient elution was achieved using a binary solvent system consisting of 10 mM ammonium bicarbonate (A), adjusted to pH 8.5 with ammonium hydroxide, and 100% of acetonitrile (B) at a flow rate of 5 μ L/min. The gradient was initiated at 98:2 (% A:B) for 5 min, followed by a linear increase to 42:58 (% A:B) at 33 min. This concentration of B was held for 3 min. Subsequently the eluent was returned to 98:2 (% A:B) at 40 min and the system was equilibrated for 10 min prior to the next injection. The mass spectrometry was performed in negative ion mode, and was scanned in the range m/z 150–2200 (2 microscans, maximum accumulation time of 150 ms, an ion count of 200,000).

2.11. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

Mass-spectrometric analysis of the oligosaccharides was performed on a 4800 Plus MALDI TOF/TOFTM (AB SCIEX) mass spectrometer. 0.8 μ L of the sample followed by 0.8 μ L of matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 70% acetonitrile/0.1% trifluoroacetic acid/water) was applied to an Opti-TOF 384 well plate. The instrument was operated in positive ion reflector mode and the m/z range from 200 to 3000 was monitored. A total of 1500 laser shots were applied to each sample in MS mode. The laser intensity was set to 4800. The MS data were analyzed manually using Data Explorer (version 4.6). For identification of 6'-Sia-GOS, the sample was incubated with 0.5 μ g of *Trypanosoma rangeli* α -2,3-sialidase mutant (Paris et al., 2005) at 37 °C in 50 mM ammonium acetate (pH 6.0) for 19 h, prior to the analysis.

3. Results

3.1. Expression and purification of PmST

The N-terminal amino acid residues 2–25 predicted to be a signal sequence by TMHMM Server v.2.0 (CBS, DTU) were excluded from the PmST gene. The N-His₆-tagged PmST was produced in *E. coli* BL21(DE3)pLysS cultured in auto-induction medium ZYM-5052. About 75 mg of soluble protein was purified from cell lysate obtained from 1 L of culture using a Ni²⁺-sepharose column. The theoretical molecular weight of the His₆-tagged PmST was 48 kDa. As displayed by SDS-PAGE (Fig. 1), the recombinant protein exhibited a single band with a similar molecular weight.

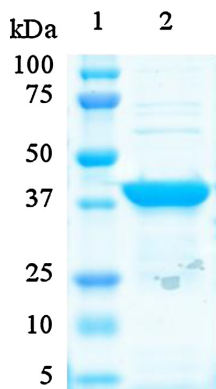


Fig. 1. SDS-PAGE of the purified N-His₆-tagged PmST. Lane 1, protein standards; lane 2, the recombinant PmST purified by affinity chromatography using a Ni²⁺-sepharose column.

3.2. Trans-sialidase activities of recombinant PmST

The trans-sialylation catalyzed by PmST (0.35 mg/mL) was performed using 10 mM pNP-Neu5Ac or 5% cGMP as the donor, and 50 mM lactose as the acceptor at pH 6.0 and 37 °C. The reactions were sampled at 0.5 h, 2 h, 4 h, 6 h and 8 h, respectively. As detected by TLC, PmST catalyzed the sialylation of lactose to form 3'-sialyllactose, but unexpectedly also 6'-sialyllactose was generated from the reaction with both pNP-Neu5Ac (Fig. 2A) and cGMP (Fig. 2B) as the donors. 3'-sialyllactose was the dominant product detectable after 0.5 h, and 6'-sialyllactose was formed at a lower rate detectable after 2 h. The two products generated from the 8-h reaction using cGMP as the donor were further confirmed as 3'-sialyllactose and 6'-sialyllactose by LC–MS analysis (Fig. S2).

3.3. Influence of the reaction parameters on 3'-sialyllactose and 6'-sialyllactose production

In order to identify the optimum reaction conditions for enzymatic production of 3'- and 6'-sialyllactoses, a statistically designed experiment was set up, considering the factors temperature (30–50 °C), pH (4.4–6.4) and lactose concentration (50–150 mM) at different reaction times (0.5 h, 2 h, 4 h, 6 h and 8 h). Further, this should probe the possibility to favor regioselective trans-sialylation.

Multiple linear regression analysis of the response surface model data showed that among the reaction factors, pH was the only factor influencing the 3'-sialyllactose synthesis. In addition,

Table 1

The optimum conditions and maximum yields for 3'- and 6'-sialyllactoses at different time points as estimated from the response surface models.^a

Time (h)	3'-Sialyllactose			6'-Sialyllactose		
	pH	T (°C)	Yield (mM)	pH	T (°C)	Yield (mM)
0.5	5.4	41	1.68	5.2	37	0.087
2	5.5	40	2.28	5.4	39	0.69
4	6.4	50	2.38	5.4	39	1.48
6	6.4	50	2.78	5.4	39	2.40
8	6.4	50	2.15	5.4	39	2.87

^a The donor was 5% cGMP (9 mM bound sialic acid), and the acceptor was 100 mM lactose.

the interaction effect for pH × temperature was significant throughout the reaction (Table S1). Curvature effects for both temperature and pH (i.e., pH × pH, temp × temp interactions) were also observed (Table S1). For the shorter reaction times (0.5 h and 2 h) (Figs. 3A and S3A; Table 1), the optimum condition for 3'-sialyllactose production was pH 5.4 and 40 °C; however, at longer reaction times (4–8 h), the optimum shifted toward the highest pH (6.4) and temperature (50 °C) tested (Figs. 3B, S3B and C; Table 1). This shift in optimum is presumed to be related to the inherently confounding effect on 3'-sialyllactose concentration resulting from an initial product build-up and subsequent consumption.

For 6'-sialyllactose production, the individual factors showed no significant effect on the yield except pH at 0.5 h, while curvature effects for pH and temperature (i.e., pH × pH, temp × temp) were significant (Table S2). At the initial stage of the reaction (0.5 h and 2 h), the interaction between pH and temperature significantly affected 6'-sialyllactose production (Table S2). The modeled pH–temperature optimum was 5.4–39 °C, similar to that observed for 3'-sialyllactose production at shorter reaction times, and remained constant between 0.5 h and 8 h with the yield increasing over time (Figs. 3C and S4; Table 1).

The summary of the fit and the predictability of the models were satisfactory with the values of Q² and R² within the range of 0.55–0.94 and 0.79–0.98, respectively, indicating reasonably trustworthy models (Tables S1 and S2).

3.4. Time course study of trans-sialylation reactions

The RSM experiments identified pH 5.4 at 40 °C as the optimum condition when considering reaction rates for the trans-sialidase activities indicating that the application of this condition and careful control of reaction time could be one way to regulate the ratio between 3'- and 6'-sialyllactoses. It also suggested, however, that changing pH to 6.4 had a more negative impact on 6'-sialyllactose than 3'-sialyllactose production indicating an alternative route for regioselective trans-sialylation.

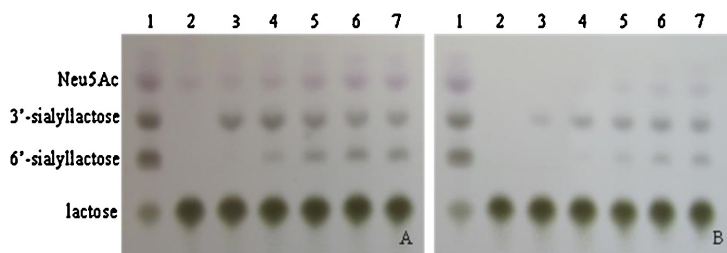


Fig. 2. TLC profile of trans-sialylation catalyzed by PmST. (A) The reaction was performed with 10 mM pNP-Neu5Ac as the donor and 50 mM lactose as the acceptor; (B) the reaction was performed with 5% cGMP (9 mM bound sialic acid) as the donor and 50 mM lactose as the acceptor. Lane 1, standards; lanes 2–7, the samples collected at 0 h, 0.5 h, 2 h, 4 h, 6 h and 8 h, respectively.

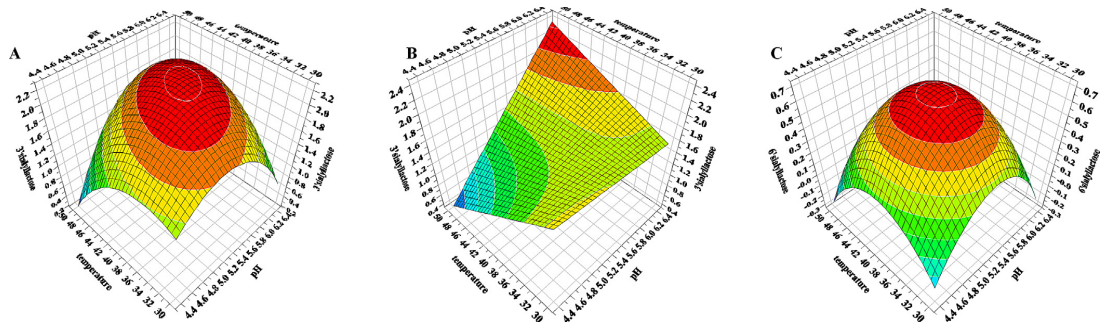


Fig. 3. Response surface plot showing the effect of pH and temperature on the production of 3'-sialyllactose (mM) at 2 h (A) and 4 h (B), and the production of 6'-sialyllactose (mM) at 2 h (C). The lactose concentration is constant at the center level (100 mM). 5% cGMP was used in all reactions.

To explore the correlations among 3'-sialyllactose, 6'-sialyllactose and sialic acid synthesis, a time course of the trans-sialylation reaction at pH 5.4 was compared to that at pH 6.4 (5% cGMP and 100 mM lactose, 40 °C). At pH 5.4 (Fig. 4A), 3'-sialyllactose was produced at a much faster rate and accumulated to a concentration of 2.42 ± 0.38 mM at 2 h, but markedly decreased afterwards. An equimolar concentration of 3'- and 6'-sialyllactoses was seen after 4 h, while the concentration of 6'-sialyllactose increased up to 3.33 ± 0.38 mM at 8 h. At this point, the product composition consisted of 65.6% 6'-sialyllactose, 16.3% 3'-sialyllactose and 18.1% sialic acid. At pH 6.4, 3'-sialyllactose was produced with a slower rate, reaching a concentration of 2.75 ± 0.35 mM at 6 h (Fig. 4B). In contrast, the 6'-sialyllactose activity was strongly inhibited at pH 6.4, with a yield of 0.67 ± 0.028 mM at 8 h. At 6 h, the product composition comprised 80.2% 3'-sialyllactose and only 14% 6'-sialyllactose and 5.8% sialic acid. The sialidase activity of PmST was much lower than its trans-sialidase activities with maximum release of sialic acid of 0.92 ± 0.059 mM and 0.27 ± 0.011 mM after 8 h at pH 5.4 and 6.4, respectively.

The trans-sialidase activities of PmST were also examined with a 20-fold lower enzyme concentration (0.0175 mg/mL) under the optimized condition (pH 5.4, 40 °C). The reaction was performed for 24 h (Fig. S5), giving rise to synthesis of 3'-sialyllactose, 6'-sialyllactose and sialic acid with yields of 0.75 ± 0.01 mM, 0.13 ± 0.0013 mM, and 0.066 ± 0.0055 mM, respectively.

3.5. Apparent kinetics of trans-sialidase activity

The kinetic parameters for the PmST-catalyzed 6'-sialyllactose synthesis were estimated by reacting the enzyme for up to 1 h under the optimized reaction condition (pH 5.4, 40 °C) on different concentrations of 3'-sialyllactose (up to 400 mM), while keeping lactose concentration constant (100 mM). The rate of the 6'-sialyllactose formation increased in response to the increase in 3'-sialyllactose concentration, but never reached saturation status (Fig. S6). By fitting the equation $v = [E_0][S_0](k_{cat}/K_m)$ to the data, the k_{cat}/K_m value was calculated to be $23.22 \pm 0.7 \text{ M}^{-1} \text{ s}^{-1}$.

3.6. Production and identification of Sia-GOS

The optimum condition for production of 6'-sialyllactose (pH 5.4–40 °C, 8 h) was applied to the reaction for sialylation of GOS, and Sia-GOS were purified by anion exchange chromatography (Fig. S7). MALDI-MS analysis showed that Sia-GOS consisted of different species having chain lengths of DP 2–6, in agreement with the observed m/z of 656.3, 818.4, 980.5, 1142.6 and 1304.6 (Fig. 5A). The purified Sia-GOS sample was also treated with an α -2,3-sialidase, and the 3'-Sia-GOS were hydrolyzed, giving rise to free sialic acid (m/z 332.1) and neutral hexose (primarily galactose and glucose) (m/z 365.1, 527.2, 689.3, 851.4 and 1013.5) as shown in Fig. 5B. The 6'-Sia-GOS (m/z 656.3, 818.4, 980.5 and 1142.6) were resistant to the treatment by sialidase-catalyzed hydrolysis.

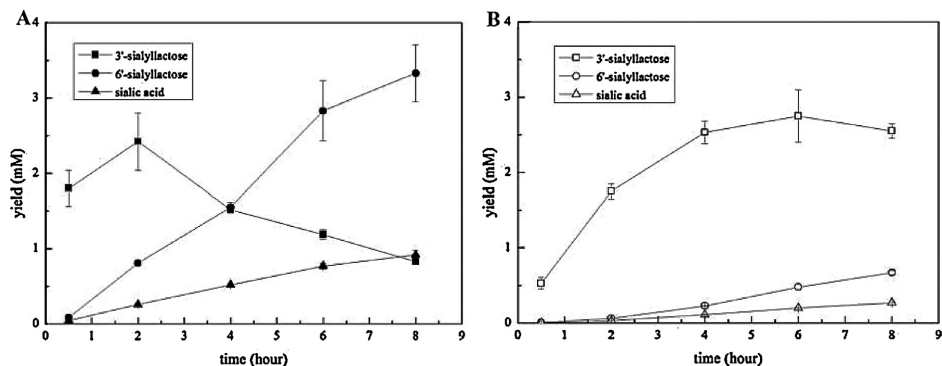


Fig. 4. Time course of production of 3'- and 6'-sialyllactoses and sialic acid. (A) The optimum condition for 6'-sialyllactose production was pH 5.4 and 40 °C; (B) the optimum condition for 3'-sialyllactose production was pH 6.4 and 40 °C. The donor was 5% cGMP, and the acceptor was 100 mM lactose. Data are given as mean \pm standard deviation, $n = 2$.

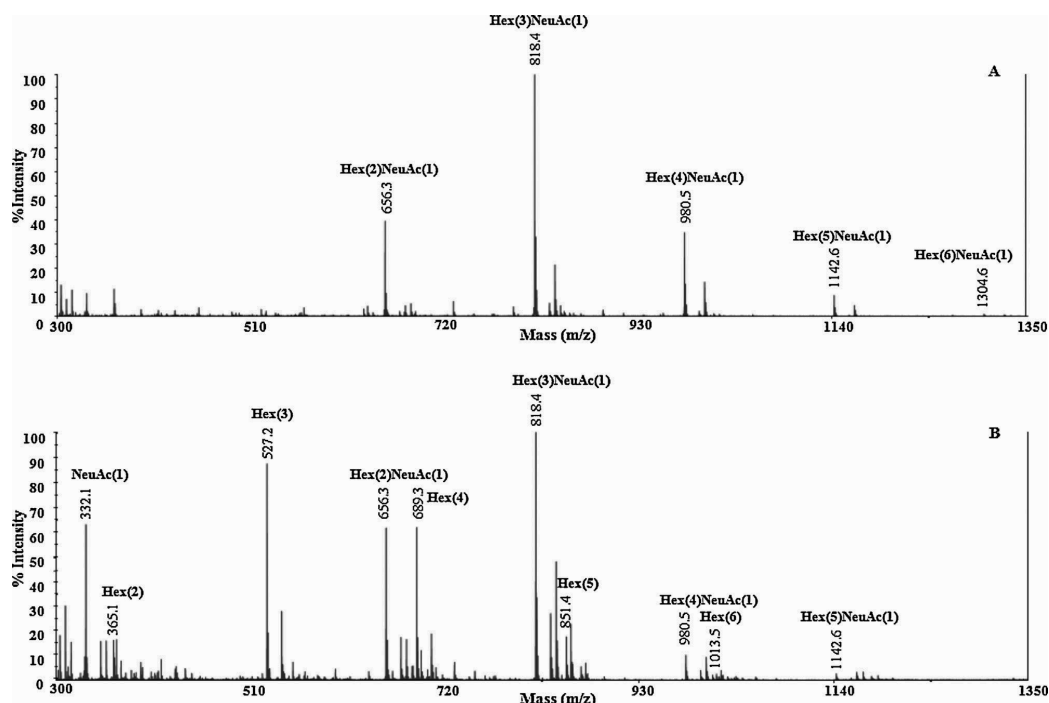


Fig. 5. MALDI-MS analysis of the purified Sia-GOS. (A) The five MS peaks represent GOS (DP 2–6) decorated with α -2,3 and α -2,6 bound sialic acid residues; (B) treatment of A with an α -2,3-sialidase, leading to a peak representing free sialic acid, five peaks representing GOS (DP 2–6), and 4 peaks representing resistant 6'-Sia-GOS (DP 2–5). Hex represents hexose (primarily galactose and glucose) residues; Neu5Ac represents sialic acid residue; values in parentheses denote the degree of polymerization of hexose moieties.

4. Discussion

Compared to tPm0188Ph (Yu et al., 2005), the recombinant PmST displayed dual trans-sialidase activities, forming products with both α -2,3 and α -2,6 sialyl linkages in the trans-sialylation. This is unlikely due to three amino acid differences among their protein sequences (N105D, Q135R, and E295G), since they are located at least 20 Å away from the active site of PmST. In comparison to the reaction conducted by Yu et al. (2005) using 0.015 mg/mL tPm0188Ph, 10 mM 3'-sialyllactose and 5 mM 4-methylumbelliferyl β -D-lactoside (LacMu) at 37 °C and pH 6.0, a 23-fold higher concentration of enzyme (0.35 mg/mL) and a 10-fold higher concentration of acceptor (50 mM lactose) were employed in our work (Fig. 2), possibly promoting the trans-sialidase activity of PmST for 6'-sialyllactose synthesis. Apart from that, the sampling time (80 min) could have been too short to observe the product with α -2,6 sialyl linkage in their work (Yu et al., 2005), since 6'-sialyllactose was detected later than 3'-sialyllactose in our reaction. This was also supported by the fact that the application of 0.0175 mg/mL PmST in the trans-sialylation reaction (pH 5.4, 40 °C) led to the production of 3'-sialyllactose with a yield of 0.12 ± 0.019 mM and 6'-sialyllactose with a barely detectable concentration (about 0.01 mM) after 2 h (Fig. S5).

Interestingly, while PmST had a lower α -2,6- compared to α -2,3-trans-sialidase activity, it also exhibited a preference for α -2,3 sialyl linkages as donor for both hydrolysis and transfer. In practice, this resulted in a slower production of 6'-sialyllactose, which was not degraded again by PmST, in contrast to 3'-sialyllactose. This was also demonstrated by the fact that a majority of 6'-sialyllactose and

free sialic acid but a very low concentration of 3'-sialyllactose were detected by HAPC after 48-h reaction (data not shown). This observation coincided with the report of Yu et al. (2005) that tPm0188Ph displayed no hydrolysis activity on Neu5Ac α -2,6LacMu. Moreover, it indicated that α -2,6 bound sialic acid in cGMP was not, or at best inefficiently, used as substrate for trans-sialylation.

To date, four SiaTs have been found to display multiple functions in addition to the native SiaT activity. Apart from tPm0188Ph and PmST in this study, a *Photobacterium damsela* α -2,6-SiaT (Δ 15Pd2,6ST) has α -2,6-trans-sialidase and α -2,6-sialidase activities (Cheng et al., 2010), and a *Campylobacter jejuni* α -2,3/2,8-SiaT (CstII) displays α -2,8-trans-sialidase and α -2,8-sialidase activities (Cheng et al., 2008). Generally, promiscuous activities share the main active-site features with the native activity (Khersonsky and Tawfik, 2010). Functioning as a SiaT, tPm0188Ph follows a displacement (inverting) mechanism, where Asp141 serves as a general base to activate acceptor hydroxyl group, and His311 might serve as a general acid and protonate the departing phosphate oxygen of CMP (Ni et al., 2006, 2007). As an α -2,3-sialidase, tPm0188Ph follows a double displacement (retaining) mechanism and it has been suggested that Asp141 is a possible general acid/base and His311 is a possible nucleophile (Sugiarto et al., 2011). Since a trans-sialidase follows a retaining mechanism similar to a sialidase, but with galactosides used as acceptors instead of water, this assumption could also be applicable for the trans-sialidase activity of tPm0188Ph as well as those of PmST in this study. In some cases, the same catalytic residue can act in a different protonation state in the native compared to the promiscuous function (Khersonsky and Tawfik, 2010). The different activities of tPm0188Ph have different

optimum pHs: the α -2,3-SiaT (pH 7.5–9.0), α -2,3-trans-sialidase (pH 5.5–6.5) and α -2,3-sialidase activities (pH 5.0–5.5), possibly being involved in modulating charges and consequently functionalities of critical residues (Sugiarto et al., 2011; Yu et al., 2005).

The PmST-catalyzed trans-sialylation using cGMP as the donor and lactose as the acceptor was a cascade reaction where the initial product of 3'-sialyllactose was converted to 6'-sialyllactose. It appeared evident that the enzyme functioned optimally at pH 5.4 and 40 °C, where the reaction rates were the fastest and both sialyllactose isomers were formed. An initial accumulation of 3'-sialyllactose was followed by its degradation. 6'-sialyllactose was produced throughout the reaction, but was not degraded and hence accumulated over time. For favoring production of either sialyllactose isomer, it was therefore key to terminate the reaction at an appropriate time. After an 8-h reaction (longest tested), a maximum yield of 6'-sialyllactose (3.33 ± 0.38 mM) and simultaneously 0.83 ± 0.019 mM 3'-sialyllactose were produced from 5% cGMP (Fig. 4A). A higher concentration of 6'-sialyllactose should be obtainable by prolonging reaction time at pH 5.4 and 40 °C until all 3'-sialyllactose is consumed. The same condition could be used for 3'-sialyllactose production; however, the reaction should be terminated in an early state (i.e., 2 h) before 3'-sialyllactose degradation became dominant. Interestingly, the RSM experiments indicated that an increase in pH to 6.4 would be detrimental to 6'-sialyllactose production while only slowing down 3'-sialyllactose production. Therefore, a more efficient method for favoring 3'-sialyllactose production was application of the condition (pH 6.4–40 °C) in a 6-h reaction that resulted in a maximum yield of 3'-sialyllactose (2.75 ± 0.35 mM) with a concomitant production of 0.48 ± 0.033 mM 6'-sialyllactose (Fig. 4B). The use of cGMP, a waste side stream from cheese production, as Neu5Ac donor was applied successfully for maximum production of total sialyllactose in a yield of 46.2% and 35.9% at the two distinct optimum conditions, respectively. In contrast, the *A. ureafaciens* sialidase-catalyzed trans-sialylation enabled sialyllactose synthesis in a yield of only 7.9–13.8% from cGMP (McJarow et al., 2003). In previous studies using another enzyme class, sialidases, for trans-sialylation also indicated the importance of reaction conditions with both temperature and reaction time being crucial for controlling product yield and regioselectivity (Ajisaka et al., 1994; Schmidt et al., 2000; Tanaka et al., 1995). It appears to be a general theme that careful examination of reaction conditions is paramount for enzymatic trans-glycosylation when using enzymes retaining hydrolytic activity, and/or having dual regioselectivities.

The kinetic study of 3'-sialyllactose conversion for 6'-sialyllactose synthesis confirmed the ability of PmST to transfer an α -2,3 bound sialic acid residue to an α -2,6 bound residue, indicating a uniquely promiscuous trans-sialidase activity of the enzyme. The limited solubility of 3'-sialyllactose made it impossible to observe saturation, therefore, only the k_{cat}/K_m value was determined. The low k_{cat}/K_m value (23.22 ± 0.7 M⁻¹ s⁻¹) also underscored the main drawback of this enzyme, namely that the catalytic efficiency for trans-sialylation is very low, thereby warranting a very high enzyme concentration.

We also examined the possibility of using PmST for sialylation of GOS. As expected, both 3'- and 6'-Sia-GOS were formed and identified by MALDI-MS. Albeit GOS are widely used for supplementation of infant formula, the structural discrepancy between GOS and HMOs renders them unlikely to mimic HMOs (Bode, 2009). Recently, 3'-Sia-GOS produced by the *T. cruzi* trans-sialidase-catalyzed trans-sialylation were suggested as HMO analogs for alternative supplementation of infant formula (Sallomons et al., 2013). However, the *T. cruzi* trans-sialidase has no α -2,6-trans-sialidase activity; thus, the PmST-catalyzed production of 6'-Sia-GOS suggested a novel option for obtaining new kinds of HMO analogs. Our study herein provides a new solution for

simultaneously or sequentially enzymatic production of 3'- as well as 6'-Sia-GOS.

In conclusion, this study showed that PmST had dual trans-sialidase activities capable of catalyzing formation of two sialyl linkages in a trans-sialylation reaction. Using cGMP as the donor and lactose as the acceptor, the application of optimized conditions combined with careful control of reaction time enabled maximum production of 3'- and 6'-sialyllactoses with different compositions. When GOS served as acceptors, PmST catalyzed the trans-sialylation to synthesize both 3'- and 6'-Sia-GOS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jbiotec.2013.11.013>.

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SUPPLEMENTARY MATERIAL

A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3'-sialyl and 6'-sialyl glycans

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Table S1. Multiple linear regression coefficients (Coeff.) and significances (*P*) of reaction parameters in the surface response models on 3'-sialyl/lactose production in the trans-sialylation reactions at 0.5 h, 2 h, 4 h, 6 h and 8 h, respectively^a.

Parameter	0.5 h		2 h		4 h		6 h		8 h	
	Coeff.	<i>P</i>	Coeff.	<i>P</i>	Coeff.	<i>P</i>	Coeff.	<i>P</i>	Coeff.	<i>P</i>
Constant	1.68	7.1×10^{-8}	2.27	2.24×10^{-12}	1.51	2.6×10^{-10}	1.41	1.56×10^{-7}	0.98	1.97×10^{-5}
pH	-0.064	0.50	0.18	1.92×10^{-3}	0.45	9.43×10^{-4}	0.52	1.15×10^{-4}	0.53	7.37×10^{-4}
<i>T</i>	0.13	0.20	-0.07	0.14	-0.15	0.18	-0.13	0.17	-0.24	0.06
lactose	-7.21×10^{-4}	0.99	0.011	0.80	0.11	0.3	0.047	0.59	0.14	0.26
pH \times pH	-0.63	3.74×10^{-3}	-0.72	3.69×10^{-6}	-	-	0.48	0.01	0.54	0.012
temp \times temp	-0.45	0.023	-0.58	2.65×10^{-5}	-	-	-0.49	0.01	-	-
pH \times temp	0.46	1.22×10^{-3}	0.55	5.05×10^{-7}	0.56	4.29×10^{-4}	0.50	3.73×10^{-4}	0.35	0.021
<i>Q</i> ²	0.59		0.94		0.55		0.73		0.59	
<i>R</i> ²	0.87		0.98		0.79		0.89		0.80	

^a The donor was 5% cGMP (9 mM bound sialic acid).

Table S2. Multiple linear regression coefficients (Coeff.) and significances (P) of reaction parameters in the surface response models on 6'-sialyllactose production in the trans-sialylation reactions at 0.5 h, 2 h, 4 h, 6 h and 8 h, respectively^a.

Parameter	0.5 h		2 h		4 h		6 h		8 h	
	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P
Constant	0.085	2.85×10^{-7}	0.69	1.9×10^{-6}	1.48	5.44×10^{-7}	2.40	6.10×10^{-7}	2.86	4.75×10^{-7}
pH	-13.4×10^{-3}	0.032	-0.024	0.65	-0.018	0.87	0.015	0.93	0.042	0.85
T	-9.57×10^{-3}	0.11	-0.049	0.37	-0.13	0.25	-0.17	0.36	-0.22	0.33
lactose	-2.76×10^{-3}	0.62	-0.026	0.63	0.033	0.77	0.029	0.87	0.089	0.68
pH \times pH	-0.044	1.09×10^{-3}	-0.51	4.86×10^{-4}	-0.89	9.45×10^{-4}	-1.41	1.26×10^{-3}	-1.66	1.18×10^{-3}
temp \times temp	-0.026	0.025	-0.43	1.71×10^{-3}	-0.60	0.012	-1.06	7.71×10^{-3}	-1.29	6.18×10^{-3}
pH \times temp	0.018	0.012	0.27	0.022	-	-	-	-	-	-
Q ²	0.66		0.63		0.58		0.60		0.60	
R ²	0.88		0.88		0.84		0.85		0.85	

^a The donor was 5% cGMP.

1 M K T I T L Y L D P A S L P A L N Q L M D F T Q N N E D K T
 atgaaaaaatcacgctgtatctggatccggcatcacttccggcactgaatcaactgatggattttacacagaacaacgaagataaaacg
 91 H P R I F G L S R F K I P D N I I T Q Y Q N I H F V E L K D
 catccgagaatttttggcctgtcaagattcaaaatcccgataacattattacacagatcagaaacattcattttgtcgaactgaaagat
 181 N R P T E A L F T I L D Q Y P G N I E L D I H L N I A H S V
 aacagaccgacagaagcgctgttttacaattctggatcaatatccgggaaacatcgaactggatccatctgaatattgcacatagcgtc
 271 Q L I R P I L A Y R F K H L D R V S I Q R L N L Y D D G S M
 caactgattagaccgattctggcgtatcgctttaaacatctggatagagtcagcattcaacgcctgaatctgtatgatgatggctcaatg
 361 E Y V D L E K E E N K D I S A E I K Q A E K Q L S H Y L L T
 gaatatgtcgatctggaaaaagaagaacaaagatatattagcgcagaaaataaacaggcggaaaaacaactgagccattatctgctgacg
 451 G K I K F D N P T I A R Y V W Q S A F P V K Y H F L S T D Y
 ggcaaaatcaaatattgataatccgacaaatcgacgctatgtttggcaatcagcatttccgggtcaaatatcattttctgagcacagattat
 541 F E K A E F L Q P L K E Y L A E N Y Q K M D W T A Y Q Q L T
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 631 P E Q Q A F Y L T L V G F N D E V K Q S L E V Q Q A K F I F
 ccggaacaacaagcgttttatctgacactggctggctttaatgatgaagttaaacaaagcctggaagtccaacaggcgaaatttatcttt
 721 T G T T T W E G N T D V R E Y Y A Q Q Q L N L L N H F T Q A
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 811 G G D L F I G D H Y K I Y F K G H P R G G E I N D Y I L N N
 ggccggagatctttttatcggcgatcattacaaaatctactttaaaaggccatccgcgtggcggagaaaataatgattatattctgaacaac
 901 A K N I T N I P A N I S F E V L M M T G L L P D K V G G V A
 gcgaaaaacattacgaacattccgggaaacattagctttgaagtccgtgatgatgacaggcctgctgcccggataaagtgtggcgagttgca
 991 S S L Y F S L P K E K I S H I I F T S N K Q V K S K E D A L
 tcactcgttatttttactgcccgaagaaaaaatcagccatcatctttacaagcaacaacagggtcaaaagcaagaagatgcgctg
 1081 N N P Y V K V M R R L G I I D E S Q V I F W D S L K Q L
 aataaccgctatgttaaagttatgcgcgactgggcattattgatgaaagccaagtcattttttgggatagccctgaaacagctg

Fig. S1. The synthetic nucleotide and protein sequences of PmST.

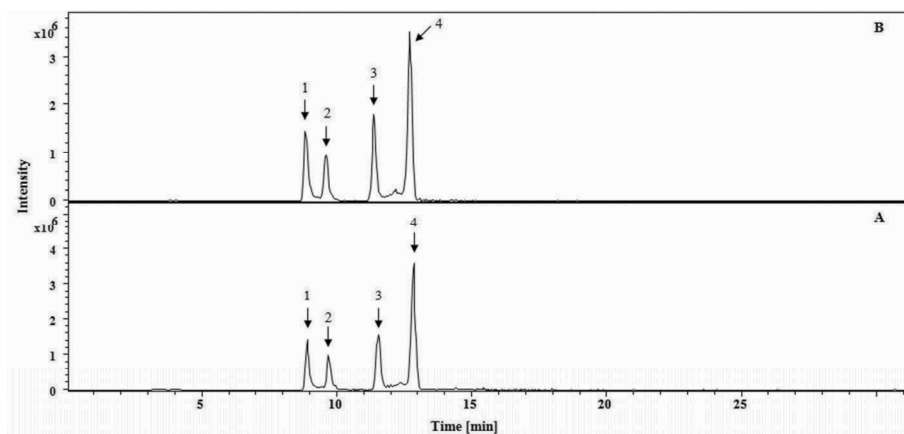


Fig. S2. LC-MS analysis of 3'- and 6'-sialyllactoses. (A) 6'-sialyllactose standard (peak 1 and 2) and 3'-sialyllactose standard (peak 3 and 4); (B) sialyllactoses formed in the trans-sialylation with cGMP as the donor and lactose as the acceptor. The split peaks represent the α - and β -isomers respectively, of the hemiacetal in the reducing end of the sialyllactoses.

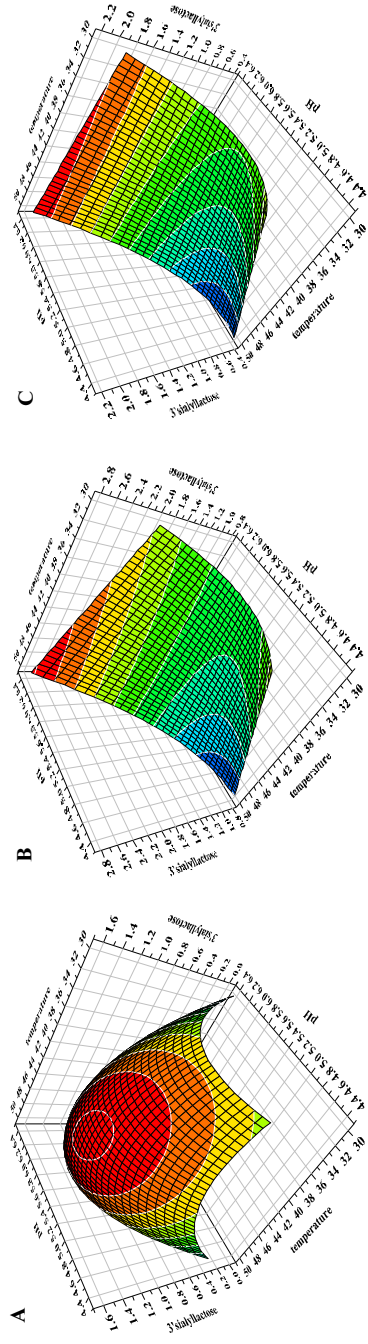


Fig. S3. Response surface plot showing the effect of pH and temperature on the production of 3'-sialyllactose (mM) at 0.5 h (A), 6 h (B) and 8 h (C). The lactose concentration is constant at the center level (100 mM). 5% cGMP (9 mM bound sialic acid) was used in all reactions.

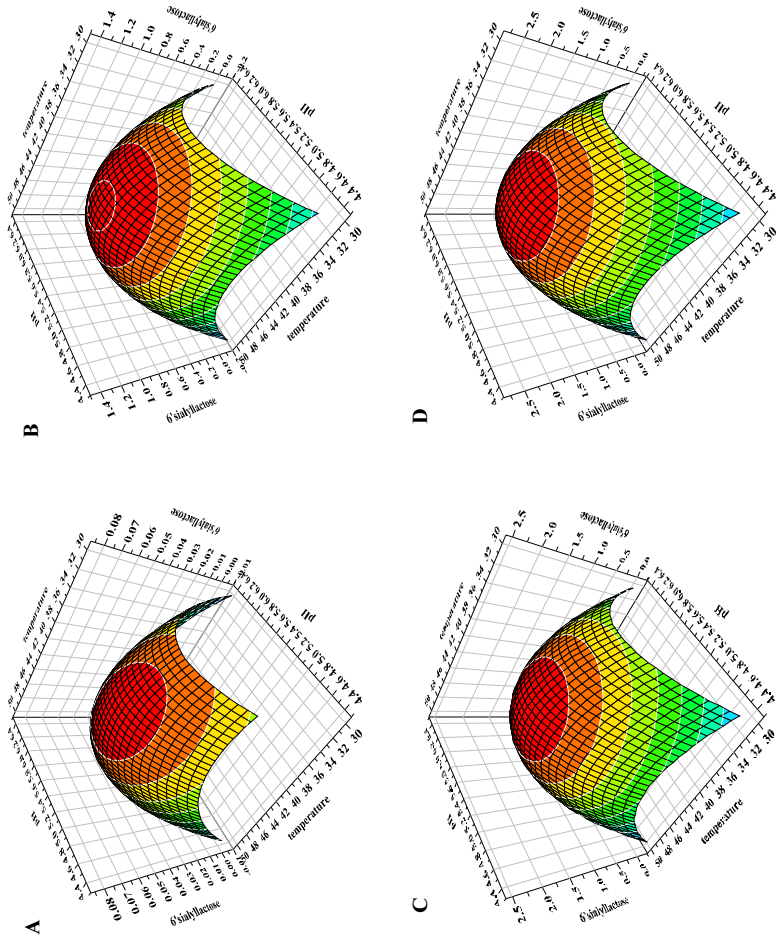


Fig. S4. Response surface plot showing the effect of pH and temperature on the production of 6'-sialyllactose (mM) at 0.5 h (A), 4 h (B), 6 h (C) and 8 h (D). The lactose concentration is constant at the center level (100 mM). 5% cGMP was used in all reactions.

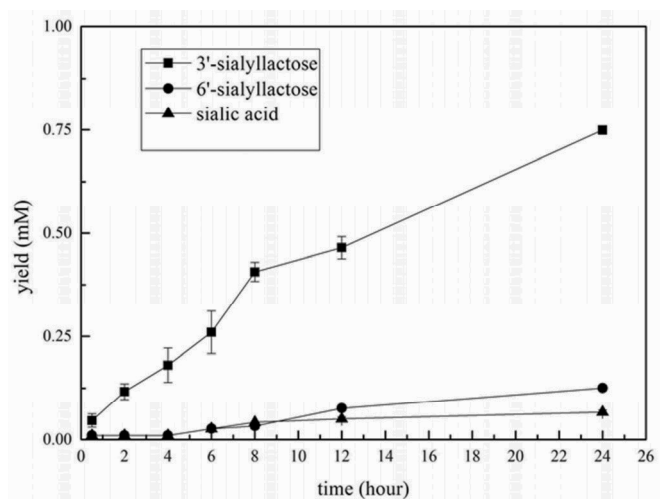


Fig. S5. Time course of production of 3'- and 6'-sialyllactoses and sialic acid. The reactions were performed with 5% cGMP as the donor and 100 mM lactose as the acceptor at pH 5.4 and 40 °C. The enzyme concentration was 0.0175 mg/mL. The samples were collected at 0.5 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h, respectively. Data are given as mean±standard deviation, n=2.

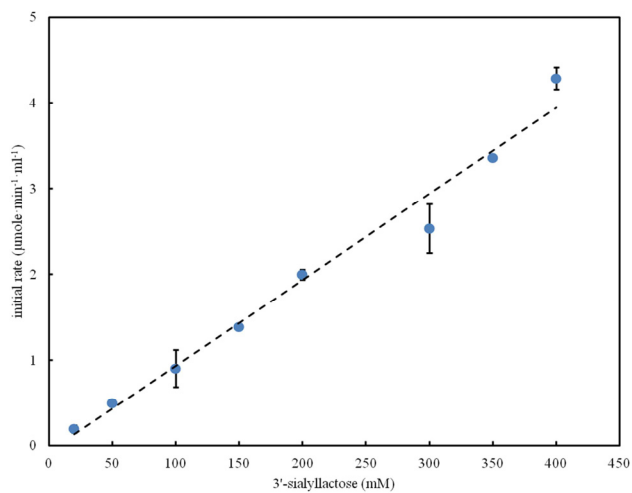


Fig. S6. Kinetic profile for α -2,6-trans-sialidase activity of PmST. The reactions were done with 20–400 mM 3'-sialyllactose and 100 mM lactose at pH 5.4 and 40 °C within 1 h. Data are given as mean \pm standard deviation, n=2.

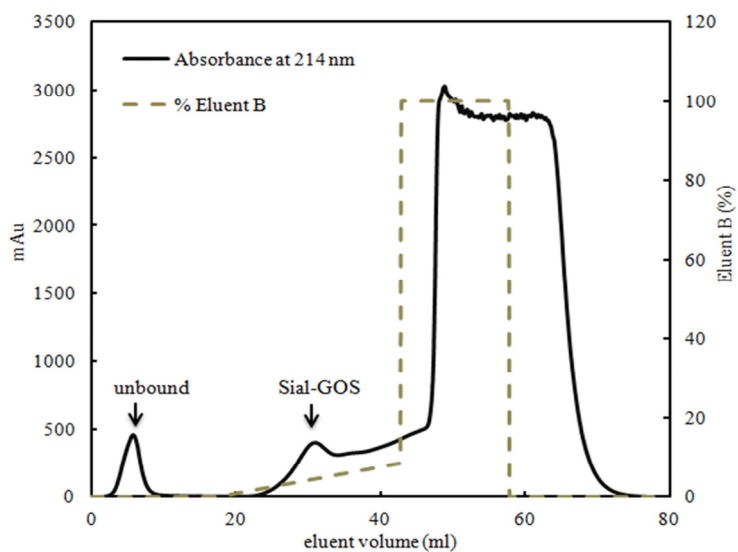


Fig. S7. Purification of Sial-GOS by anion exchange chromatography using a sepharose column. Eluent B was 1 M ammonium formate.

Modulating the regioselectivity of *Pasteurella multocida* sialyltransferase for biocatalytic production of 3'- and 6'-sialyllactoses

Authors

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Abstract

This study examined the trans-sialylation efficacy and regioselectivity of five designed mutants of *Pasteurella multocida* sialyltransferase (PmST) catalysing the synthesis of 3'-sialyllactose and 6'-sialyllactose using casein glycomacropeptide as the sialyl-donor and lactose as the acceptor. The mutant enzymes PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y} preferentially catalysed synthesis of 3'-sialyllactose over 6'-sialyllactose. When a total sialyllactose (3'- and 6'-sialyllactose) yield of ~50% was obtained from casein glycomacropeptide, the regioisomeric ratio (α -2,3: α -2,6) and the transfer ratio, *i.e.* total sialyllactose:sialic acid, obtained from a reaction catalysed by the best mutant PmST_{E271F/R313Y} were 64 and 33, respectively. These values were 128- and 5-folds higher than those obtained by use of the wild type enzyme (PmST_{WT}), respectively. Another mutant enzyme PmST_{P34H} displayed a distinct preference for synthesis of 6'-sialyllactose during the entire course of reaction. Although the total sialyllactose yield was consistently significantly lower than that of PmST_{WT}, the (α -2,6: α -2,3) ratio was 1.3-77-fold higher than that obtained by PmST_{WT} catalysis during the first 8 h of the reaction, and reached a maximum of 13.4 after 8 h. The PmST_{P34H} mutant produced a 980-fold increase in α -2,6-sialyltransferase activity compared to PmST_{WT}, and the α -2,3-sialyltransferase activity of PmST_{P34H} was almost abolished. The k_{cat}/K_m value for PmST_{P34H} using 3'-sialyllactose as the donor for 6'-sialyllactose synthesis at pH 5.4 and 40 °C was 31.2 M⁻¹ s⁻¹ (the k_{cat}/K_m of PmST_{WT} was 23.2 M⁻¹ s⁻¹). Both PmST_{WT} and PmST_{P34H} were also capable of catalysing the hydrolysis and transfer of α -2,6 bound sialic acid.

Keywords

Pasteurella multocida sialyltransferase; site-directed mutagenesis; regioselectivity; 3'-sialyllactose; 6'-sialyllactose

1 Introduction

Sialylated human milk oligosaccharides (HMOs) consist of *N*-acetylneuraminic acid (Neu5Ac) attached to galactose (Gal) or *N*-acetylglucosamine (GlcNAc) through α -2,3 and/or α -2,6 sialyl linkage (Bode, 2009). During lactation, the content of 6'-sialyllactose (6'-SL) gradually declines while that of 3'-SL remains relatively stable (Wang, 2009); nevertheless, the content of 6'-SL is at least double that of its isomer (Coppa et al., 1999; Gabrielli et al., 2011; Leo et al., 2010). For monosialylated lacto-*N*-tetraoses (LNTs), the content of sialyl-lacto-*N*-tetraose c (LST c, α -2,6 bound sialic acid) is also higher compared to that of LST a (α -2,3 bound sialic acid) especially in

colostrum (Coppa et al., 1999; Gabrielli et al., 2011; Leo et al., 2010). The α -2,3- and α -2,6-sialylated HMOs have individual biological functions. For instance, 6'-SL was effective in inhibiting the *Pseudomonas aeruginosa* invasion of pneumocytes (Marotta et al., 2014), and 3'-SL and 3'-sialyl-3-fucosyl-lactose were identified to inhibit leukocyte rolling and adhesion to endothelial cells (Bode et al., 2004).

Examples of large-scale biocatalytic synthesis of sialylated HMOs and analogues thereof, *e.g.*, SL, LST, and sialylated galactooligosaccharides (Sia-GOS) from casein glycomacropeptide (cGMP) are given with use of *Arthrobacter ureafaciens* or *Bifidobacterium infantis* sialidase (Mcjarrow et al., 2003), *Trypanosoma cruzi* trans-sialidase (Holck et al., 2014; Pelletier et al., 2004; Sallomons et al., 2013), or *Trypanosoma rangeli* sialidase mutants (Jers et al., 2014; Michalak et al., 2014). However, most produced sialyloligosaccharides contain exclusively α -2,3 bound sialic acid residues. In the previous study (Guo et al., 2014), we have reported that a *Pasteurella multocida* sialyltransferase (PmST) exhibits α -2,3/2,6-trans-sialidase activities. When this enzyme was examined for catalysing the sialylation of lactose using cGMP as donor substrate, we identified reaction conditions that favoured production of either 3'-SL or 6'-SL. For 6'-SL, a mixture of isomers was produced with initial build-up and degradation of 3'-SL and a slower accumulation of 6'-SL and therefore the time of reaction termination determined the fraction of 6'-SL in the reaction.

Protein engineering can be an effective tool for altering the regioselectivity of glycosidases and glycosyltransferases (GTs) (Choi et al., 2008 and 2014; Dion et al., 2001; He et al., 2006; Hellmuth et al., 2008). The objective of the work was to address the options for controlling the regioselectivity of PmST via site-directed mutagenesis, notably for more efficient α -2,6-trans-sialylation, and meanwhile attempt to understand the significance of specific, selected point mutations in the enzyme. Previously, four mutants (E271F, R313Y, E271F/R313Y and M144D) derived from a PmST homologue enzyme (Δ 24PmST1) have been described, of which the α -2,3-sialidase activities were reduced by 59-6333 folds without affecting α -2,3-sialyltransferase (α -2,3-SiaT) activity (Sugiarto et al., 2011 and 2012). Our hypothesis was that these mutants - if less affected in trans-sialidase activity - might prove better catalysts compared to PmST wild type (PmST_{WT}) for production of sialylated glycans. Moreover, in bacterial SiaTs from GT family 80, the residue P34 in PmST_{WT} is replaced with a His residue in α -2,6-SiaTs. Based on structural studies, this His residue has been suggested to be a key factor for determining α -2,6-regiospecificity in α -2,6-SiaTs (Kakuta et al., 2008). Hence, as an additional strategy, we introduced the point

mutation P34H by site-directed mutagenesis with the aim of altering the regioselectivity towards α -2,6-trans-sialylation.

2 Materials and Methods

2.1 Chemicals and reagents

Phusion DNA polymerase, T4 DNA ligase, *Nco*I, *Eco*31I and *Kpn*I were purchased from Thermo Scientific (Waltham, US). Protein standards for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, US). Cytidine-5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac), 3'- and 6'-SL sodium salts, and 3'- and 6'-sialyl-*N*-acetylglucosamine (SLN) sodium salts were purchased from Carbosynth (UK). The commercial cGMP product LACPRODAN CGMP-20 was supplied by Arla Foods (Viby, DK). The Neu5Ac content was 0.18 mmole per gram of cGMP. Before use, impurities of low molecular weight in the cGMP solution were removed by filtration through Ultracel regenerated cellulose membranes (5 kDa cutoff) (Merck Millipore, US). All other chemicals were purchased from Sigma-Aldrich (St. Louis, US).

2.2 Enzyme mutagenesis, expression and purification

The gene encoding PmST_{WT} (Genbank accession number AAK02272) was previously inserted in pETM-11 (EMBL, DE) and expressed in *Escherichia coli* BL21(DE3)plysS (Novagen, USA) (Guo et al., 2014). The gene encoding mutant PmST_{P34H} was PCR amplified using PmST_{WT} gene as template and primers P34H_F/WT_R (Table S1). The PCR product was digested with *Eco*31I and *Kpn*I and inserted in pETM-11 between *Nco*I and *Kpn*I in frame with an N-terminal His₆-tag (N-His₆-tag). PmST_{R313Y} was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, US) with PmST_{WT} gene as template and primers R313Y_F/R313Y_R (Table S1). PmST_{E271F} and PmST_{M144D} were generated using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific, US) with the recombinant plasmid pETM11-PmST_{WT} as template and primers E271F_F/E271F_R and M144D_F/M144D_R (Table S1) respectively. Double mutant PmST_{E271F/R313Y} was generated using the same kit with pETM11-PmST_{R313Y} as the template and primers E271_F/E271_R. The resulting plasmids were used to transform *E. coli* BL21(DE3)plysS for expression. Expression and purification of the wild type and mutant enzymes were carried out using the method described previously (Guo et al., 2014). Fractions containing N-His₆-tagged proteins were collected and then desalted using Vivaspin 6 (10 kDa cutoff) (Sartorius AG, DE) to

remove imidazole. Protein concentrations were determined using a Bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific) with BSA as standard.

2.3 Trans-sialylation assay

All trans-sialylation reactions were carried out in duplicate or triplicate using 100 mM lactose as the acceptor at 40 °C in 50 mM citrate-phosphate buffer (pH 5.4) (optimum identified previously) (Guo et al., 2014). Samples were taken at various time points, inactivated by heating at 90 °C for 10 min and analysed by HPAEC-PAD as described below. 1 unit (U) of trans-sialidase activity was defined as the enzyme amount that catalysed the transfer of 1 μ mole Neu5Ac to lactose per minute at the assay condition described. 1 U of hydrolysis activity was defined as the enzyme amount that catalysed the hydrolysis of 1 μ mole Neu5Ac per minute at the assay condition described.

Using 5% (w/v) cGMP (9 mM bound sialic acid) as the donor, a reaction was initiated by addition of 0.35 mg/mL hydrolase mutants (PmST_{E271F}, PmST_{R313Y}, PmST_{E271F/R313Y}, and PmST_{M144D}) and 1.05 mg/mL PmST_{P34H} mutant, respectively. For comparison, 0.35 mg/mL or 1.05 mg/mL PmST_{WT} was used in a benchmark reaction. All reactions proceeded for 12 h. To examine pH effect on PmST_{P34H}-catalysed trans-sialylation, reactions were performed for 20 h in 50 mM citrate-phosphate buffer with a pH range of 4.4-7.4.

Using 1 mM 3'-SLN as the donor to follow 3'-SL formation, a reaction was initiated by addition of 0.023 mg/mL PmST_{WT} or PmST_{E271F}, 0.07 mg/mL PmST_{R313Y} or PmST_{E271F/R313Y}, and 1.05 mg/mL PmST_{P34H}, respectively. To follow Neu5Ac release during the trans-sialylation reaction, separate reactions using 1.05 mg/mL PmST_{WT} or mutant enzymes were performed, respectively. All reactions proceeded for 0.5 h.

Using 1 mM 3'-SL as the donor to follow 6'-SL formation and Neu5Ac release, a reaction was carried out using 0.53 mg/mL enzyme (PmST_{WT}, PmST_{E271F}, PmST_{R313Y}, PmST_{E271F/R313Y} or PmST_{P34H}). Additionally, 1.05 mg/mL PmST_{P34H} was used to follow the Neu5Ac release during the reaction. All reactions proceeded for 2 h. The kinetic assay for PmST_{P34H}-catalysed α -2,6-trans-sialylation was done using the method described previously (Guo et al., 2014). Reactions were initiated by addition of 0.53 mg/mL PmST_{P34H}, and the initial velocity was measured within 1 h. The k_{cat}/K_m value was derived from a linear fit $v=[E_0][S_0](k_{cat}/K_m)$.

Using 1 mM 6'-SLN as the donor to follow 6'-SL formation and Neu5Ac release, a reaction was carried out using 1.05 mg/mL PmST_{WT} (2 h) and 0.35 mg/mL PmST_{P34H} (4 h), respectively. Additionally, 1.05 mg/mL PmST_{P34H} was used to follow the Neu5Ac release during the reaction.

Using 1 mM 6'-SL as the donor to follow 3'-SL formation and Neu5Ac release, a reaction was carried out using 1.05 mg/mL PmST_{WT} or PmST_{P34H} for 2 h.

2.4 Sialidase assay

Using 2 mM pNP-Neu5Ac as the substrate, the sialidase assay was done in triplicate in 50 mM citrate-phosphate buffer (pH 7.6) at 40 °C. A reaction was initiated by addition of 0.35 mg/mL PmST_{P34H}, 5.25 mg/mL PmST_{M144D}, and 1.75 mg/mL PmST_{WT}, PmST_{E271F}, PmST_{R313Y} or PmST_{E271F/R313Y}, respectively. Hydrolysed pNP was monitored continuously at 400 nm ($\epsilon=11577 \text{ cm}^{-1}\text{M}^{-1}$) for 1 h using a Tecan Infinite M200 spectrophotometer (Männedorf, CH). 1 U of sialidase activity was defined as the enzyme amount that catalysed the hydrolysis of 1 μmole pNP per minute at the assay condition described.

Using 1 mM 3'-SL as the substrate, a reaction was carried out for 0.5 h with use of 0.07 mg/mL enzyme (PmST_{WT}, PmST_{E271F}, PmST_{R313Y}, PmST_{E271F/R313Y} or PmST_{P34H}). Using 1 mM 6'-SL as the substrate, a reaction was carried out using 0.35 mg/mL PmST_{P34H} (2 h), 1.05 mg/mL PmST_{WT} or PmST_{E271F} (2 h), 1.05 mg/mL PmST_{R313Y} (4 h) and 1.05 mg/mL PmST_{E271F/R313Y} (8 h), respectively. All reactions were done at pH 5.4 and 40 °C in duplicate or triplicate. Samples were withdrawn at various time points, inactivated by heating at 90 °C for 10 min and analysed by HPAEC-PAD as described below. 1 U of hydrolysis activity was defined as the enzyme amount that catalysed the hydrolysis of 1 μmole Neu5Ac per minute at the assay condition described.

2.5 Sialyltransferase assay

α -2,3-sialyltransferase and α -2,6-sialyltransferase assays were performed at their corresponding optimum pH conditions identified by Yu et al. (2005). An α -2,3-sialyltransferase assay was performed in 50 mM Na₂HPO₄-KH₂PO₄ buffer (pH 8.34) using 1 $\mu\text{g/mL}$ PmST_{WT} (2 h) and PmST_{M144D} (1 h), respectively. An α -2,6-sialyltransferase assay was carried out in 50 mM citrate-phosphate buffer (pH 5.4) using 50 $\mu\text{g/mL}$ PmST_{WT} (1 h) and 1 $\mu\text{g/mL}$ PmST_{P34H} (0.5 h), respectively. All reactions were done in duplicate or triplicate at 37 °C using 2 mM CMP-Neu5Ac as the donor and 100 mM lactose as the acceptor. Samples were taken at various time points, inactivated by heating at 95 °C for 5 min, and analysed by HPAEC-PAD as described below. 1 U of SiaT activity was defined as the enzyme amount that catalysed the transfer of 1 μmole Neu5Ac to lactose per minute at the assay condition described.

2.6 Analytical methods

All samples from sialidase, trans-sialylation and sialyltransferase reactions were measured by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). After termination of reactions, reaction mixtures were centrifuged (5 min at 20,000 g) and supernatants were filtered through Vivaspın 500 (5 kDa cutoff) (Sartorius). Concentrations of 3'-SL, 6'-SL and Neu5Ac in permeates were quantified as described previously (Guo et al., 2014). The separation programme was modified by addition of a 5-min cleaning step using 100 % of 500 mM sodium acetate prior to re-equilibration, in order to remove impurities.

Samples from PmST_{P34H}-catalysed trans-sialylation reaction using 5% cGMP and 100 mM lactose as substrates (pH 5.4-40 °C, 12 h) were also analysed by capillary liquid chromatography/mass spectrometry (LC-MS) as described previously (Guo et al., 2014).

3 Results

3.1 Production of PmST mutant enzymes

The five N-His₆-tagged PmST mutant enzymes (PmST_{E271F}, PmST_{R313Y}, PmST_{E271F/R313Y}, PmST_{M144D} and PmST_{P34H}) were successfully expressed in *E. coli* BL21(DE3)plysS with a yield of 70-80 mg of enzyme purified from 1 L of culture. The enzymes had the expected molecular weight of ~48 kDa (Fig. 1).

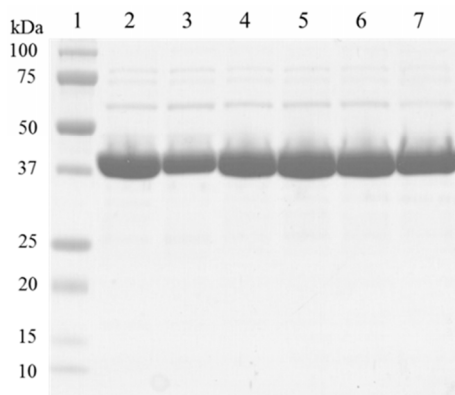


Fig. 1 SDS-PAGE of purified N-His₆-tagged PmST wild type and mutant enzymes. Lane 1, protein standards; lane 2-7, recombinant PmST_{WT}, PmST_{P34H}, PmST_{E271F}, PmST_{R313Y}, PmST_{E271F/R313Y} and PmST_{M144D} purified by affinity chromatography using a Ni²⁺-sepharose column.

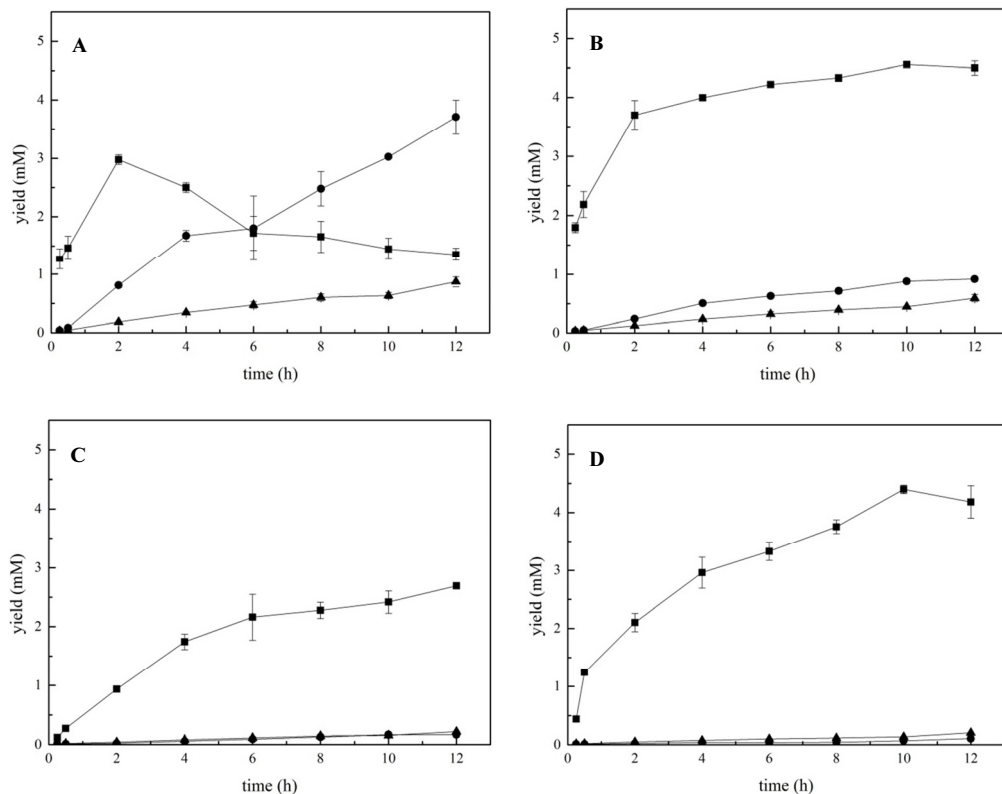


Fig. 2 Time course of production of 3'-SL (■), 6'-SL (●) and Neu5Ac (▲) in trans-sialylation reactions catalysed by PmST_{WT} (A), PmST_{E271F} (B), PmST_{R313Y} (C) and PmST_{E271F/R313Y} (D). Reactions were performed with 5% cGMP (9 mM bound sialic acid) and 100 mM lactose as the substrates at pH 5.4 and 40 °C. Samples were collected at 0.25 h, 0.5 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h respectively. Data are given as mean±standard deviation, $n=2$.

3.2 Comparison of hydrolase mutants for trans-sialylation

The PmST_{M144D} mutant exhibited only very low trans-sialidase activity (Fig. S1), but was verified to have SiaT activity maintained (data not shown). For the other three mutants (PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y}), the comparison of the trans-sialylation during 12 h showed that the activity for 6'-SL production was decreased and the hydrolytic sialidase activity was also reduced as compared to PmST_{WT} (Fig. 2). Hence, the three mutants designed to have decreased hydrolysis activity all exhibited a preferential production of 3'-SL with very low (<1 mM) yields of 6'-SL produced. The mutants PmST_{E271F} and PmST_{E271F/R313Y} produced similar yields of 3'-SL (~4.5

mM) after 10 h, and these yields were superior to that of PmST_{WT} and represented a clear change in regioselectivity towards 3'-SL production. This product selectivity was very different from the characteristic product profile evolution for the PmST_{WT}-catalysed trans-sialylation, which involved an initial selectivity for 3'-SL production, but a concomitant production of 6'-SL, and a reversal between the yields of 6'-SL and 3'-SL during extended reaction (Fig. 2A).

When total SL yields of ~50% were obtained (~32% for PmST_{R313Y}), regioisomeric ratios (α -2,3: α -2,6) for PmST_{WT} (10 h), PmST_{E271F} (4 h), PmST_{R313Y} (12 h), and PmST_{E271F/R313Y} (10 h) were 0.5, 7.9, 16, and 63.9 respectively (Table S2), corroborating the superior regioselectivity of the PmST_{E271F/R313Y} for α -2,3-sialylation. Meanwhile, transfer ratios (total SL:Neu5Ac) for PmST_{WT}, PmST_{E271F}, PmST_{R313Y}, and PmST_{E271F/R313Y} were 7.1, 19.2, 13.2, and 33.3 respectively, once again underscoring that PmST_{E271F/R313Y} was the better enzyme for trans-sialylation. For PmST_{R313Y}, the overall (α -2,3: α -2,6) ratios and transfer ratios were better than those of PmST_{WT}, but this mutant produced lower SL yields compared to the other enzymes (Table S2).

Table 1 Comparison of α -2,6-SiaT activity among bacterial α -2,6-SiaTs from GT family 80.

Enzyme	CMP-Neu5Ac (mM)	Lactose (mM)	pH	<i>T</i> (°C)	Specific activity (U/mg)	Reference
<i>P. damsela</i> α -2,6-SiaT	2.8	50	5	30	7	Yamamoto et al., 1998
<i>P. leiognathi</i> α -2,6-SiaT	2.8	50	5	30	3.81	Yamamoto et al., 2007
<i>Photobacterium</i> sp. Δ 16pspST6	2.3	120	6	30	113	Tsukamoto et al., 2008
<i>Photobacterium</i> sp. Δ 109pspST6	2.3	120	6	30	264	Kakuta et al., 2008
<i>P. leiognathi</i> α -2,6-SiaT	2.3	120	7	30	82.9	Mine et al., 2010
PmST _{WT}	2	100	5.4	37	0.02	This study
PmST _{P34H}	2	100	5.4	37	19.6	This study

3.3 Regioselective synthesis of 6'-SL

It was previously suggested that the His residue (corresponding to P34 in PmST) in bacterial α -2,6-SiaTs might determine α -2,6-regiospecificity (Kakuta et al., 2008). Here, we assessed the effect of the point mutation P34H on the native SiaT activities of the enzyme. Strikingly, PmST_{P34H}

acquired a much higher (980-fold) α -2,6-SiaT activity than PmST_{WT} at pH 5.4 (Table 1), and this increase in α -2,6-SiaT activity was accompanied by loss of α -2,3-SiaT activity at both pH 5.4 and pH 8.34 (data not shown). Next, we wanted to test the applicability of this mutant for regioselective α -2,6-trans-sialylation.

In fact, the PmST_{P34H} mutant demonstrated a significant preference for 6'-SL production with very low levels of 3'-SL produced and Neu5Ac released. The products from the trans-sialylation reaction were verified as 6'- and 3'-SLs by LC-MS analysis (Fig. S2). The α -2,6-trans-sialidase activity of this mutant was very low with 1.4 mM 6'-SL obtained after 10 h (Fig. 3B). The corresponding PmST_{WT}-catalysed reaction, *i.e.* with use of a high enzyme concentration (3-fold higher than that used in Fig. 2A), the yield of 3'-SL (2.9 mM) peaked after a shorter reaction time (0.5 h). After 6 h, 0.4 mM 3'-SL was retained while the 6'-SL concentration increased to 4.5 mM (Fig. 3A). The highest ratio of (α -2,6: α -2,3) was 13.4 and 15.8 for PmST_{P34H} and PmST_{WT} with a total SL yield of 15% and 53.1% respectively (Table S3). Thus, the direction of PmST_{P34H}-catalysed trans-sialylation was altered toward 6'-SL production, but at the price of a low yield.

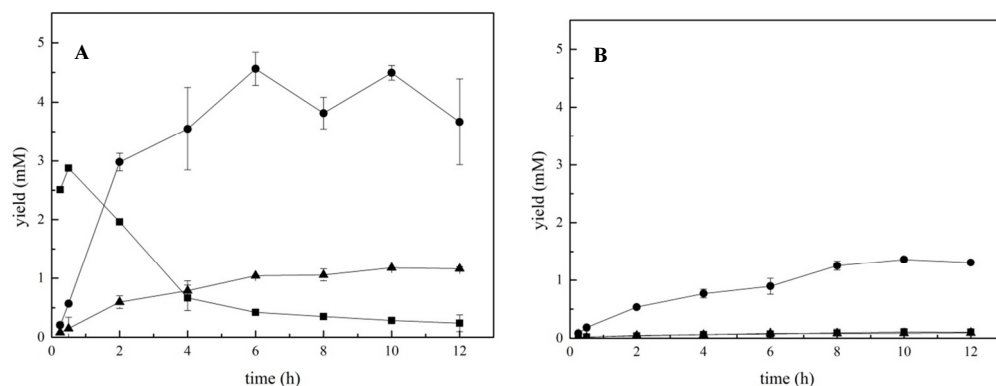


Fig. 3 Time course of production of 3'-SL (■), 6'-SL (●) and Neu5Ac (▲) in trans-sialylation reactions catalysed by PmST_{WT} (A) and PmST_{P34H} (B). Reactions were performed with 5% cGMP and 100 mM lactose as the substrates at pH 5.4 and 40 °C. Samples were collected at 0.25 h, 0.5 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h respectively. Data are given as mean±standard deviation, *n*=2.

We previously demonstrated that α -2,6-trans-sialidase activity of PmST_{WT} was optimal at pH 5.4 and strongly inhibited at pH 6.4 in an 8-h reaction (Guo et al., 2014). Here, the effect of pH on the PmST_{P34H}-catalysed trans-sialylation was examined in the pH range of 4.4-7.4. The data obtained indicated that pH had a similar effect on the PmST_{P34H}-catalysed synthesis of 3'-SL and 6'-SL (Figs.

4A and B). 6'-SL was produced at the highest rate at pH 5.4, but reached a plateau at ~1.3 mM after about 8 h. In contrast, at pH 6.4 a lower rate was observed, but the accumulation of 6'-SL continued in the 20-h reaction, reaching a final concentration of 1.95 mM. The reason why the reaction at pH 5.4 appeared to be inactivated was not further explored. Thus, shifting pH from 5.4 to 6.4 led to a decrease in reaction rate, but an increase in total SL yield and little change in regioselectivity.

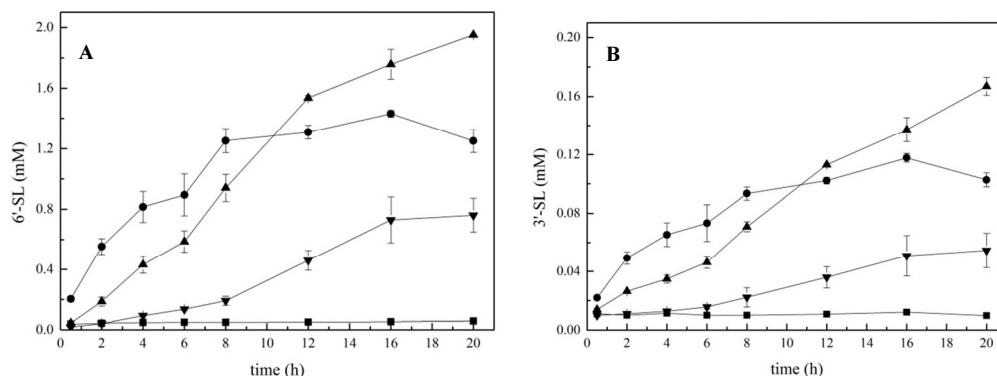


Fig. 4 pH effect on PmST_{P34H}-catalyzed trans-sialylation. Reactions were performed with 5% cGMP and 100 mM lactose as the substrates at 40 °C in a citrate-phosphate buffer (■, pH 4.4; ●, pH 5.4; ▲, pH 6.4; ▼, pH 7.4). Samples were collected at 0.5 h, 2 h, 4 h, 6 h, 8 h, 12 h, 16 h and 20 h respectively. (A) pH effect on production of 6'-SL; (B) pH effect on production of 3'-SL. Data are given as mean±standard deviation, *n*=2.

3.4 Comparison of trans-sialidase and sialidase activities among PmST_{WT} and the derived mutants

The impact of donor linkage on the rate of product formation was also investigated. As it is not trivial to isolate the individual activities, our approach was to use 3'-SL as donor to monitor 6'-SL production and vice versa. Further, we used the alternative donor 3'-SLN and 6'-SLN to follow production of 3'-SL and 6'-SL respectively. Conversely, the trans-sialylation rate for production of the other isomer was ignored. In the trans-sialylation reaction with use of 3'-SLN as the donor for 3'-SL production, PmST_{E271F} maintained its α -2,3-trans-sialidase activity while PmST_{R313Y} and PmST_{E271F/R313Y} retained 77% and 67% α -2,3-trans-sialidase activity respectively, as compared to PmST_{WT} (Table 2). The reduction in α -2,3-trans-sialidase activity of these three mutant enzymes was less than that in sialidase activity thereof (Table 2). In the experiment using 3'-SL as the donor for production of 6'-SL, it was apparent that PmST_{WT} exhibited a 23-fold higher α -2,3- compared to α -2,6-trans-sialidase activity. Interestingly, the mutants, especially PmST_{E271F/R313Y}, displayed a

larger reduction in α -2,6- compared to α -2,3-trans-sialidase activity (Table 2), effectively shifting the specificity towards α -2,3-trans-sialylation as observed (Fig. 2).

Table 2 Comparison of sialidase and trans-sialidase activities among PmST wild type and mutant enzymes. Reactions were done using 1 mM 3'-sialyl donor at pH 5.4 and 40 °C. 100 mM lactose was used in trans-sialidase reactions. Data are given as mean \pm standard deviation, $n=3$.

Enzyme	Substrate	3'-SLN and lactose		3'-SL and lactose		α -2,3-: α -2,6-transfer
		α -2,3-transfer (U/g)	Hydrolysis (U/g)	α -2,6-transfer (U/g)	Hydrolysis (U/g)	
PmST _{WT}		129 \pm 20 (100) ^a	1.3 \pm 0.2 (100)	5.6 \pm 0.3 (100)	1.2 \pm 0.1 (100)	23
PmST _{E271F}		132 \pm 21 (100)	0.8 \pm 0.1 (59)	2.3 \pm 0.3 (41)	0.6 \pm 0.07 (53)	58
PmST _{R313Y}		100 \pm 1 (77)	0.5 \pm 0.1 (35)	0.5 \pm 0.05 (8)	0.3 \pm 0.05 (27)	212
PmST _{E271F/R313Y}		87 \pm 14 (67)	0.3 \pm 0.1 (24)	0.2 \pm 0.02 (3)	0.2 \pm 0.04 (14)	466
PmST _{P34H}		0.3 \pm 0.04 (0.2)	^b	5.6 \pm 0.8 (100)	^b	0.05

^a Numbers in parentheses indicate relative activity as compared to that of PmST_{WT}.

^b The activity was too low to be accurately determined.

In the case of PmST_{P34H}, the ability to use α -2,3 bound sialic acid as the donor for α -2,3-trans-sialylation was almost abolished, whereas the use of this donor for α -2,6-trans-sialylation was at a level comparable to PmST_{WT}. This was further confirmed by kinetical analysis, where PmST_{P34H} exhibited a similar behaviour as PmST_{WT}, *i.e.*, no saturation was reached in PmST_{P34H}-catalysed α -2,6-trans-sialylation (Fig. S3). The k_{cat}/K_m value for 6'-SL production was calculated to be 31.2 \pm 4.9 M⁻¹ s⁻¹, 1.3-fold of that of PmST_{WT}.

Next, we tested the applicability of α -2,6 bound sialic acid as donor for the PmST_{WT} and PmST_{P34H} (Table 3; Fig. S4). When 6'-SLN served as the donor, PmST_{P34H} displayed an α -2,6-trans-sialidase activity for 6'-SL synthesis which was 17-fold higher than that of the PmST_{WT}. Using 6'-SL as the donor, PmST_{P34H} had a 2-fold higher α -2,3-trans-sialidase activity for 3'-SL synthesis than PmST_{WT}. In both cases, the activities were very low as compared to the α -2,3-trans-sialidase activity of PmST_{WT}.

In addition, when 3'-SL, 6'-SL or pNP-Neu5Ac was used as a substrate in a sialidase reaction, the mutant enzymes generally displayed decreased sialidase activities as compared to PmST_{WT} (Table S4), albeit with the donors and reaction conditions employed here we failed to reproduce the very large reduction in hydrolase activity reported previously for PmST_{E271F}, PmST_{R313Y}, and

PmST_{E271F/R313Y} (Sugiarto et al., 2011 and 2012). For PmST_{P34H} a higher sialidase activity (>4-fold) for 6'-SL hydrolysis as well as for pNP-Neu5Ac hydrolysis was observed.

Table 3 Comparison of sialidase and trans-sialidase activities between PmST_{WT} and PmST_{P34H}. Reactions were done using 1 mM 6'-sialyl donor at pH 5.4 and 40 °C. 100 mM lactose was used in trans-sialidase reactions. Data are given as mean±standard deviation, *n*=3.

Substrate Enzyme	6'-SLN and lactose		6'-SL and lactose	
	α -2,6-transfer (U/g)	Hydrolysis (U/g)	α -2,3-transfer (U/g)	Hydrolysis (U/g)
PmST _{WT}	0.1±0.02 (100) ^a	^b	0.1±0.02 (100)	0.1±0.02
PmST _{P34H}	2.1±0.2 (1688)	^b	0.23±0.03(202)	^b

^a Numbers in parentheses indicate relative activity as compared to that of PmST_{WT}.

^b The activity was too low to be accurately determined.

4 Discussion

It has been known that donor and acceptor specificity, and reaction conditions can be employed to control or partially control the regioselectivity for enzymatic trans-sialylation (Ajisaka et al., 1994; Guo et al., 2014; Schmidt et al., 2000; Tanaka et al., 1995; Thiem and Sauerbrei, 1991). The study herein demonstrated a possibility of modulating the regioselectivity of PmST for α -2,3- or α -2,6-trans-sialylation by means of site-directed mutagenesis. By application of three hydrolase mutants, especially PmST_{E271F/R313Y}, a significant increase in the regioisomeric ratio (α -2,3: α -2,6) as well as in transfer ratio could be achieved without loss of SL yield in extended trans-sialylation reactions using cGMP and lactose as the substrates (Table S2). The increase in regioselectivity of the three mutants towards α -2,3-trans-sialylation did not appear to be attributed to the decrease in sialidase activity, but rather to a larger reduction in α -2,6-trans-sialidase activity (Fig. 2; Table 2). Interestingly, the point mutation E271F led to 2-fold reduction in sialidase as well as α -2,6-trans-sialidase activity, but had no influence on α -2,3-trans-sialidase activity. Sequence alignment of STs from GT family 80 showed that E271 in PmST_{WT} corresponded to the residue Thr in α -2,3-STs and to Ala in α -2,6-STs (Fig. S5). However, the structure of Δ 24PmST1 in complex with CMP-3F(e)Neu5Ac (PDB code 2IHK) indicated that this residue is located far away (>7 Å) from the Gal part of lactose and thus makes it difficult to rationalize its importance for α -2,3-trans-sialylation. For PmST_{R313Y} and PmST_{E271F/R313Y}, a 1.3-1.5-fold reduction in α -2,3-trans-sialidase activity was observed, while a 12.5-fold and 33-fold reduction in α -2,6-trans-sialidase activity was observed, respectively (Table 2). This effect would change the regioselectivity of the two mutants, especially

of PmST_{E271F/R313Y} towards α -2,3-trans-sialylation as observed (Figs. 2C and D). Further, a recent study of Choi et al. (2014) reported that for the PmST homologue enzyme (Δ 24PmST1), the mutants of R313N/T/Y/H/T had a large reduction in α -2,6-SiaT activity on CMP-Neu5Ac with lactose as the acceptor, and in turn an improved α -2,3-SiaT activity, corroborating the importance of R313 in modulating the regioselectivity for α -2,3-trans-sialylation. In contrast, single mutations at L433 to L433S/T in *Photobacterium damsela* α -2,6-SiaT produced 3-fold increase in the α -2,6-SiaT activity relative to the wild type enzyme activity (Choi et al., 2014). These results, in addition to the data presented in the present work, underline that it is indeed possible to limit one type of sialyl linkage synthesis while favoring another one by use of targeted single-point (or double-point) enzyme mutations. This provides significant options for rational optimisation of SiaTs for large-scale biocatalytic production of sialylated compounds.

Reduction of the hydrolase activity is also an important parameter since this should reduce degradation of sialylated products. The mutants were reported previously to exhibit a 59-6333 fold reduction in α -2,3-sialidase activity on the substrate Neu5Ac α -2,3LacMU (Sugiarto et al., 2011 and 2012). Using the substrate 3'-SL we demonstrated a 2-fold reduction in sialidase activity for the most sialidase activity impaired mutant PmST_{E271F/R313Y} (Table S4).

The structures of PmST_{WT}, PmST_{P34H} and *Photobacterium* sp. JT-ISH-224 α -2,6-SiaT in complex with lactose are shown in Fig. S6. In the active site of PmST_{P34H}, only N85 had contact with O6 of the Gal part of lactose through a hydrogen bond, while the point mutation P34H could introduce a new hydrogen bond interaction with the Gal part and thus help to restrict the regioselectivity towards α -2,6-trans-sialylation. The mutation P34H exerted the most profound effect on modulating the regioselectivity for the native SiaT activity of the enzyme, as this mutation led to a 980-fold increase in α -2,6-SiaT activity to a level that was comparable to other bacterial α -2,6-SiaTs from GT family 80 (Table 1). When tested in trans-sialylation reactions, PmST_{P34H} lost most of the activity for transfer and hydrolysis of α -2,3 bound sialic acid (Table 2). However, PmST_{P34H} maintained the activity for transfer of α -2,3 bound sialic acid to form the α -2,6 sialyl linkage, which was demonstrated by the fact that the k_{cat}/K_m value for PmST_{P34H}-catalysed α -2,6-trans-sialylation was similar to that of PmST_{WT}. When tested for trans-sialidase activity using cGMP as the donor substrate, a much lower yield of 6'-SL was obtained by PmST_{P34H} catalysis as compared to PmST_{WT}. Whether this was due to *e.g.* reduced specific activity, a pH-dependent inactivation effect (Fig. 4) or the absence of 3'-SL to function as a secondary donor is unclear. The latter would indicate that α -2,3 bound sialic acid in cGMP was not preferred as donor over 3'-SL for

α -2,6-trans-sialylation. The activity for PmST_{P34H} using α -2,6 bound sialic acid to form α -2,6 sialyl linkage was increased by 17-fold but the overall activity remained low (Table 3). The data obtained nevertheless expands the available knowledge regarding control of regioselectivity for enzymatic trans-sialylation by point mutation, and notably that a single mutation, namely P34H significantly enhanced the regioselective synthesis of α -2,6 sialyl linkages in the sialyltransferase reaction. At present, this single mutant has little applicability in trans-sialylation reactions, but represents an interesting candidate for further mutagenesis to refine selectivity and improve the reaction rate and stability. The change may involve only one base change in the triplet codon (CCT to CAT), hinting that even minor mutations may have a profound effect on the evolution of regioselectivity of bacterial SiaTs in nature.

In conclusion, this study showed that application of three mutant enzymes (PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y}) in trans-sialylation reactions enabled improved regioselective production of 3'-SL as well as diminished Neu5Ac release. Secondly, the PmST_{P34H} mutant was capable of catalysing regioselective synthesis of α -2,6 sialyl linkage in both trans-sialylation and sialyltransferase reactions.

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SUPPLEMENTARY MATERIAL

Modulating the regioselectivity of *Pasteurella multocida* sialyltransferase for biocatalytic production of 3'- and 6'-sialyllactoses

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Table S1. Primers used for site-directed mutagenesis. Primers P34H_F/WT_R, M144D_F/M144D_R, and R313Y_F/R313Y_R were used for generation of PmST_{P34H}, PmST_{M144D} and PmST_{R313Y} respectively. Primers E271F_F/E271F_R were used for generation of PmST_{E271F} and PmST_{E271F/R313Y}.

Primer	Sequence (5'-3')
P34H_F	ATCGGGTCTCCCATGAAAACAATCACGCTGTATCTGGATCATG <u>CAT</u> CACTTCCGGC ^a
WT_R	CGGGGTACCTTACAGCTGTTTCAGGCTATCCCAA ^a
M144D_F	TGATGATGGCTCAGATGAATATGTGCGATC ^{b,c}
M144D_R	TACAGATTCAGGCGTTGAATGCTGAC ^c
E271F_F	CACAACAACATGGT <u>TTT</u> GGCAATACAGATG ^{b,c}
E271F_R	CCTGTAAAGATAAAATTTGCCTGTTGGAC ^c
R313Y_F	AAGGCCATCCGTATGGCGGAGAAATTAATGATTATATTCTGAACAACGC ^b
R313Y_R	GCGTTGTTTCAGAATATAATCATTAAATTTCTCCGCC <u>ATA</u> CGGATGGCCTT ^b

^a Restriction sites *Eco31I* and *KpnI* are shown in italic.

^b Codons containing introduced changes are underlined.

^c Nucleotides at 5'-ends are phosphorylated.

Table S2. Summary of the production of 3'-SL, 6'-SL and Neu5Ac in trans-sialylation reactions catalysed by PmST_{WT}, PmST_{E271F}, PmST_{R313Y} and PmST_{E271F/R313Y}. Reactions were performed with 5% eGMP (9 mM bound sialic acid) and 100 mM lactose as the substrates at pH 5.4 and 40 °C. Samples were collected at 0.25 h, 0.5 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h respectively. The values for regioisomeric ratio (α -2,3: α -2,6) and transfer ratio (total SL:Neu5Ac) obtained when a total SL yield reached ~50% (~32% for PmST_{R313Y}) are shown in bold.

Time (h)	PmST _{WT} ^a			PmST _{E271F} ^a			PmST _{R313Y} ^a			PmST _{E271F/R313Y} ^a		
	α -2,3: α -2,6	transfer	total SL	α -2,3: α -2,6	transfer	total SL	α -2,3: α -2,6	transfer	total SL	α -2,3: α -2,6	transfer	total SL
0.25	30.4	35.7	14.6	51.8	51.2	20.4	8.7	6.7	1.5	37.4	25.2	5.0
0.5	17.3	34.6	17.2	42.5	50.3	24.9	20.8	14.1	3.1	92.0	64.8	14.0
2	3.7	20.7	42.1	15.5	31.6	43.7	40.9	22.5	10.7	95.9	44.5	23.6
4	1.5	12.1	46.4	7.9	19.2	50.0	30.4	21.7	20.0	72.3	41.0	33.4
6	1.0	7.4	39.1	6.7	15.1	53.9	23.7	19.9	25.0	93.2	33.6	37.4
8	0.7	6.9	46.0	6.0	12.8	56.1	17.7	16.7	26.7	88.0	32.3	42.2
10	0.5	7.1	49.7	5.2	12.1	60.5	14.5	16.8	28.7	63.9	33.3	49.7
12	0.4	5.8	56.2	4.9	9.1	60.2	16.0	13.2	31.8	39.3	20.7	47.7

^a 0.35 mg/mL enzyme was used in the reaction.

Table S3. Summary of the production of 3'-SL, 6'-SL and Neu5Ac in trans-sialylation catalysed by PmST_{WT} and PmST_{P34H}. Reactions were performed with 5% cGMP and 100 mM lactose as the substrates at pH 5.4 and 40 °C. Samples were collected at 0.25 h, 0.5 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h respectively. Maximal values for regioisomeric ratio (α -2,6: α -2,3) and the corresponding transfer ratio and total SL yield are shown in bold.

Time (h)	PmST _{WT} ^a			PmST _{P34H} ^a		
	α -2,6: α -2,3	transfer ratio	total SL yield (%)	α -2,6: α -2,3	transfer ratio	total SL yield (%)
0.25	0.08	<u>33.5</u>	30.2	6.2	6.3	1.1
0.5	0.2	23.0	38.3	7.5	11.1	2.3
2	1.5	8.3	54.9	12.1	12.1	6.4
4	5.3	5.3	46.8	11.9	13.2	9.2
6	10.8	4.8	55.4	12.2	11.6	10.8
8	10.8	3.9	46.3	13.4	15.8	15.0
10	15.8	4.1	53.1	12.9	16.5	16.3
12	15.3	3.4	43.4	12.8	15.0	15.7

^a 1.05 mg/mL enzyme was used in the reaction.

Table S4. Comparison of sialidase activity among PmST_{WT} and derived mutants. Reactions were done using 1 mM 3'- or 6'-SL (pH 5.4, 40 °C) and 2 mM pNP-Neu5Ac (pH 7.6, 40 °C) as the substrates respectively. Data are given as mean±standard deviation, *n*=3.

Enzyme	3'-SL (U/g)	6'-SL (U/g)	pNP-Neu5Ac (U/g)
PmST _{WT}	104±7 (100) ^a	0.4±0.04 (100)	0.06±0.00 (100)
PmST _{E271F}	86±14 (83)	0.1±0.03 (19)	0.03±0.00 (54)
PmST _{R313Y}	86±9 (83)	0.03±0.01 (8)	0.02±0.00 (37)
PmST _{E271F/R313Y}	55±6 (53)	^b	0.01 ±0.00 (25)
PmST _{P34H}	4±0.6 (4)	1.8±0.2 (444)	0.2±0.01 (434)
PmST _{M144D}	^c	^c	0.0005±0.0001 (1)

^a Numbers in parentheses indicate relative hydrolysis activity as compared to that of PmST_{WT}.

^b The activity was too low to be accurately determined.

^c Reactions were not performed.

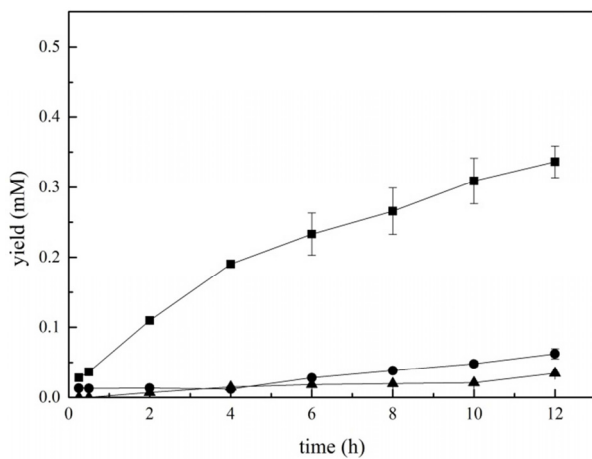


Fig. S1. Time course of production of 3'-SL (■), 6'-SL (●) and Neu5Ac (▲) using PmST_{M144D}. Reactions were done using 5% cGMP and 100 mM lactose as the substrates at pH 5.4 and 40 °C. The enzyme concentration was 1.05 mg/mL. Samples were collected at 0.5 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h, respectively. Data are given as mean±standard deviation, $n=2$.

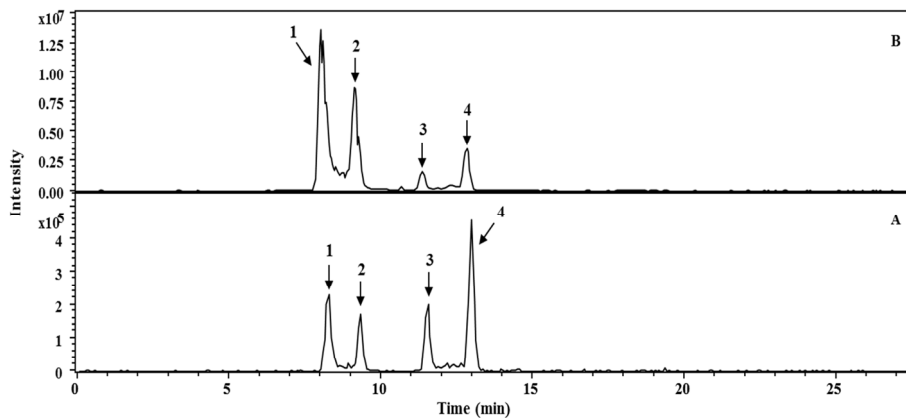


Fig. S2. LC-MS analysis of 3'- and 6'-SLs produced by PmST_{P34H} catalysis. (A) 6'-SL standard (peak 1 and 2) and 3'-SL standard (peak 3 and 4); (B) SLs formed in the 12-h trans-sialylation reaction with 5% cGMP and 100 mM lactose as substrates (pH 5.4-40 °C). The split peaks represent the α - and β -isomers respectively, of the hemiacetal in the reducing end of the SLs.

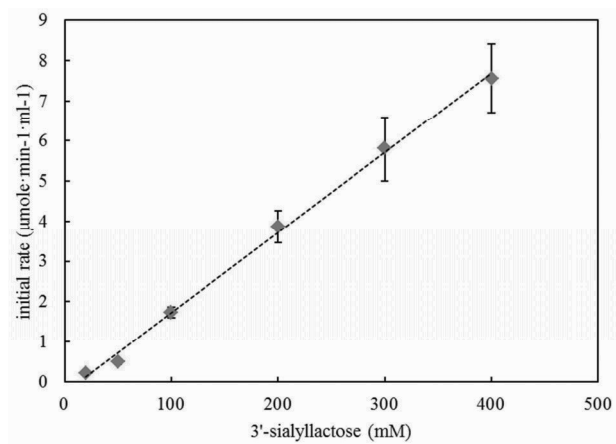


Fig. S3. Kinetic profile for α -2,6-trans-sialidase activity of PmST_{P34H}. Reactions were done with 20-400 mM 3'-SL and 100 mM lactose at pH 5.4 and 40 °C within 1 h. 0.53 mg/mL enzyme was used for each reaction. Data are given as mean \pm standard deviation, $n=3$.

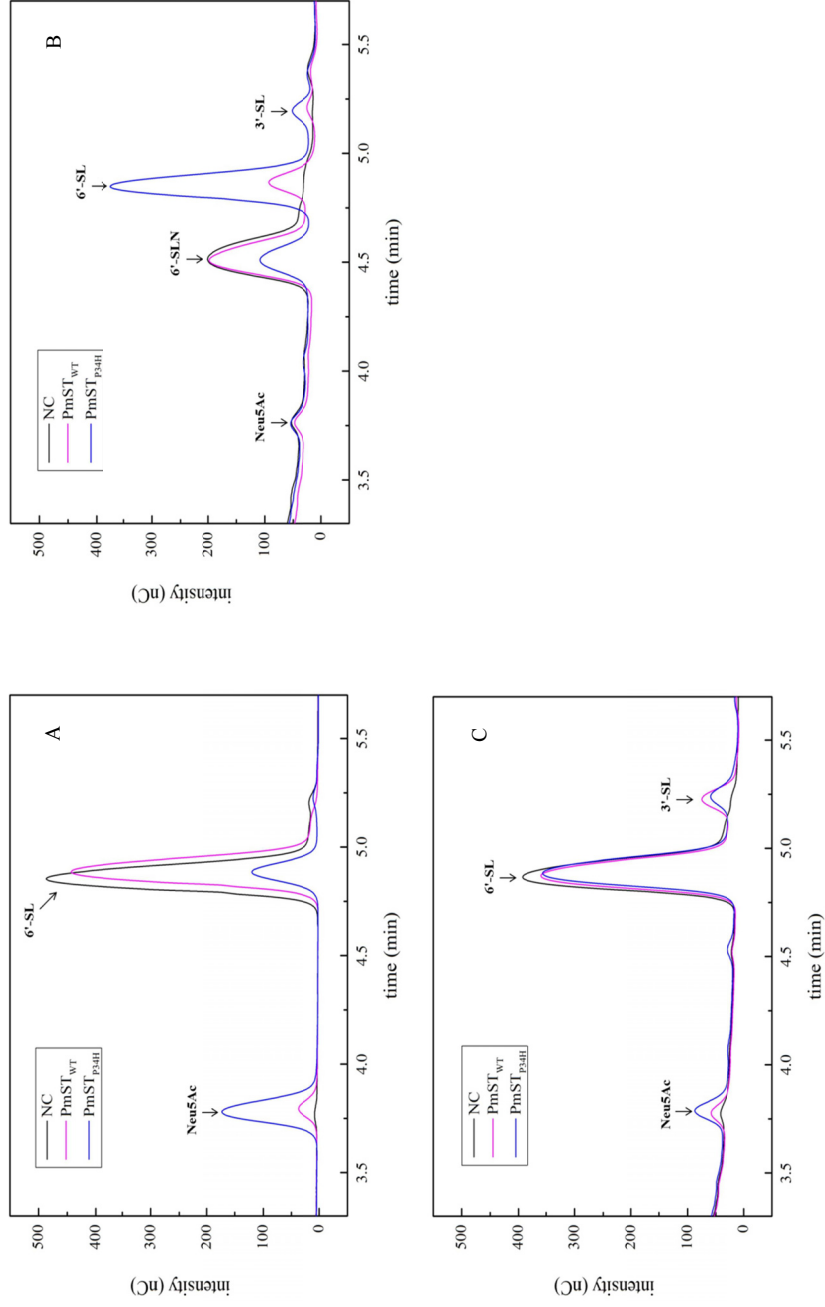


Fig. S4. HPAEC profile of reaction mixture. (A) Reactions were done using 1 mM 6'-SL as the substrate and 0.35 mg/mL PmST_{WT} (or 1.05 mg/mL PmST_{P34H}) for 4 h. (B) Reactions were done using 1 mM 6'-SLN and 0.35 mg/mL PmST_{WT} (or 1.05 mg/mL PmST_{P34H}) for 20 h. (C) Reactions were done using 1 mM 6'-SL and 100 mM lactose as the substrates and 1.05 mg/mL PmST_{WT} (or PmST_{P34H}) for 8 h. For negative control (NC) reaction, inactive enzyme (heated at 99 °C for 20 min) was used. All reactions were performed at pH 5.4 and 40 °C.

		130	140	150	160	170	180	190	200	210	220	230	240
PmST	
PI-SHI2145-a2, 6SiaT	31	YIDFASLAL	NQJLDTQ-N	NEDKTRFIF	GLSEKIPDN	IITQYQNHF	VELKDRPTE	ALFTILDQVP	GN---	TELDI	HUNTAHQQL	IRPILAYRFK	HLDRVSIQRL
Ph-ISH224-a2, 6SiaT	117	YVDIASLPSL	QQLMKIIQOK	NEYSQNERFI	SHGRILRATED	NAEKLN-AHI	YELAGNNQSQ	EUDVADIVDA	DSQURNLNEL	NNNGHSFRN	IAPILR-ATS	SNNLLISNI	NLDTGSSAEY
Ph-ISH224-a2, 6SiaT	120	YVDIASLPTL	QQLADIIKSE	BENFTAQRII	ANGRIYPTDE	QURGEL-ITS	FALKNNHTFA	DIVQEIVTQA	QURHNLNVKL	SSNTAHSPDN	LYPTIK-ELN	SFNNAVTVNI	DLTIDGSSAEY
Pd-0160-a2, 6SiaT	117	YIDIASLPSL	QQLHIIQAK	DEYFSNQRFV	SKRSVTPDAD	NANKLN-IHT	YFLKGNNTSP	EMVAADIEVA	QSKURLNTEF	YNTTARVFN	LEPIIQ-PLY	NNEKVKISHI	SLYIDGSSAEY
Ph-ISH224-a2, 3SiaT	44	YVDIATLPTI	QQMPOIINEN	SNN---	KKLI	SHSRYPINDE	ELLESINGSF	FNNN-----	S	ELIISDSMI	LNDIKWII	NGNTIARVDN	VNIKS--IE
Fp-ISH467-a2, 3SiaT	44	YVDIATLPTI	QQMPOIINEN	SNN---	KKLI	SHSRYPINDE	TILLESINGSF	FNNN-----	P	ELIISDSMI	LNDIKWII	NGNTIARVDN	VNIKS--IE
Vi-FAJ16-a2, 3SiaT	38	YVDIATLPTI	QQMCKIVSQK	TSN---	KKLI	SHSRYPITDK	SLANKINAEF	FHEQ-----	F	ELTESLNKI	LSENIDNII	HGNTLNSIDV	VDIIE--VN
		250	260	270	280	290	300	310	320	330	340	350	360
PmST	
PI-SHI2145-a2, 6SiaT	147	VDLKEKND	ISAEIIQAOK	QLSHYLLAGK	IKFON--FTI	ARYVQSQAP	VKYHFSLTDY	FEKAELQPL	KEYLAENQK	KMTFAQQLT	PEQQAIVLTL	VGFN--DEVK	QSLVEQKAF
Ph-ISH224-a2, 6SiaT	235	VELNNKQDTL	NKSQKUSQSF	LVLMQYANGI	SEER-NGYI	SYNNHQIHF	SYFFLRNDY	LEVTKLHDL	REYLGSGSLQ	KMDTFQSLS	KQDKELFLNI	VQDFQEKQK	EYQSGELNPF
Pd-0160-a2, 6SiaT	238	VNLNWRQDTL	NKTNLKIKGT	DTYLEDYNGI	NEDTSVGTQS	SVYVQKILYF	ADYFLRNDY	ROYLGSGSLQ	KMDTFQSLS	KQDKELFLNI	VQDFQEKQK	EYQSGELNPF	
Ph-ISH224-a2, 3SiaT	154	VALYDSKLP	ESEQVKISL	SDNLLISSIN	GTQPFNVVE	NIYGFQSLYF	TYTHMLRADI	FETNLFIASL	KQVLSNNIKQ	KMDYFNFEN	SQQKDFYNF	TGFNPELME	QYKASPNKF
Fp-ISH467-a2, 3SiaT	154	VALYDSKLP	ESEQVKISL	SDNLLISSIN	GTQPFNVVE	NIYGFQSLYF	TYTHMLRADI	FETNLFIASL	KQVLSNNIKQ	KMDYFNFEN	SQQKDFYNF	TGFNPELME	QYKASPNKF
Vi-FAJ16-a2, 3SiaT	148	VAIYFESKLP	ESEQKUKTSL	SNNINFSID	GTDSFNNTIE	NIYGFQSLYF	TYTHMLRADI	FETNLFIASL	KQVLSNNIKQ	KMDYFNFEN	SQQKDFYNF	TGFNPELME	QYKASPNKF
		370	380	390	400	410	420	430	440	450	460	470	480
PmST	
PI-SHI2145-a2, 6SiaT	263	IFTQTTTWAG	NTDYREYIAQ	QQMLLNHFT	QAGCDLFIG-	-DHYKIYFKG	EPFGGRINDY	ILANNKNTN	IPANISFEVL	MTGGLLPDKV	GCVASSLIVS	LPKFKISHII	FTSNKQVSK
Ph-ISH224-a2, 6SiaT	354	VFTQTTTWAG	-GETNEYIAQ	QQVNVNNAI	NETSPYILG-	-REHDLFFKG	EPFGGIINDI	ILGSGFNMD	IPAKVSFEVL	MTGGLPDTV	GCIASSLIVS	IPAKVSFIV	FTSSDITDR
Pd-0160-a2, 6SiaT	358	VFTQTTTWAG	-NHREYIAQ	QQVNVNNAI	NESSPYILG-	-NSTYDLFFKG	EPFGGIINDI	IMQNYFSND	IPSKISFEVL	MTMDLPAV	AGIASSLIVT	IPAKIKFIV	FTSTETITDR
Ph-ISH224-a2, 3SiaT	274	IFVGTNSGTA	-----TAE	QQIDILTEAK	NENSPILKS	IQGDLFFKG	EPAGGVINDI	ILGSGFNMD	IPAKISFEVL	MTMDLPAV	AGIASSLIVT	IPAKVNFIV	FTSSDITDR
Fp-ISH467-a2, 3SiaT	274	IFVGTNSGTA	-----TAE	QQIDILTEAK	NENSPILKS	IQGDLFFKG	EPAGGVINDI	ILGSGFNMD	IPAKISFEVL	MTMDLPAV	AGIASSLIVT	IPAKVNFIV	FTSSDITDR
Vi-FAJ16-a2, 3SiaT	268	IFIGNSGTA	-----TAE	QQIDILTEAK	NENSPILKS	IQGDLFFKG	EPAGGVINDI	ILGSGFNMD	IPAKISFEVL	MTMDLPAV	AGIASSLIVT	IPAKVNFIV	FTSSDITDR

Fig. S5. Multiple alignments among bacterial SiaTs in GT family 80. Protein sequences from PmST (accession No. NP_245125), *P. leiognathi* α -2,6-SiaT (accession No. BAF91416), *Photobacterium* sp. α -2,6-SiaT (accession No. BAF92026), *P. damsela* α -2,6-SiaT (accession No. BAA25316), *Photobacterium* sp. α -2,3-SiaT (accession No. BAF92025), *P. phosphoreum* α -2,3-SiaT (accession No. BAF63530), and *Vibrio* sp. α -2,3-SiaT (accession No. BAF91160) were aligned using BioEdit 7.1. The conserved residues Asp and His functioning as a general base and a possible general acid respectively for sialyltransferase activity are boxed. The residues mutated in PmST (P34, M144, E271 and R313) and their corresponding residues in other SiaTs were shaded.

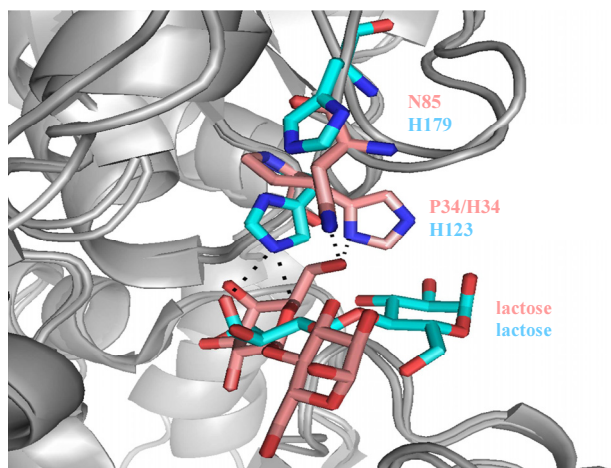


Fig. S6. Superposition among the structures of PmST_{WT}, PmST_{P34H} and *Photobacterium* sp. JT-ISH-224 α -2,6-SiaT (Δ 16pspST6, PDB code 2Z4T) in complex with lactose. Modelled structures of PmST_{WT} and PmST_{P34H} were obtained by auto-mated homology modeling using Swiss-Model (Arnold et al., 2006; Schwede et al., 2003) based on the structure of Δ 24PmST1-CMP-lactose (PDB code 2ILV). P34 from PmST_{WT}, H34 from PmST_{P34H} and N85 from PmST_{P34H} are shown in salmon, while the corresponding residues H123 and H179 from Δ 16pspST6 are shown in cyan. Bound lactose molecules from PmST_{P34H} and Δ 16pspST6 are shown in salmon and cyan, respectively. Atoms of oxygen, nitrogen, fluoride are shown in red, blue and orange respectively. Hydrogen-bond interactions with H123 and bound lactose in Δ 16pspST6, and the interactions with N85, H34 and lactose in PmST_{P34H} are shown in dark dashes.

Reference

Arnold K., Bordoli L., Kopp J., Schwede T., 2006. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modeling. *Bioinformatics* 22,195-201.

Schwede T., Kopp J., Guex N., and Peitsch M.C., 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research* 31, 3381-3385.

3. SITE-SATURATION MUTAGENESIS OF *MICROMONOSPORA VIRIDIFACIENS* SIALIDASE

This chapter is extended in the form of paper III.

3.1 Hypotheses and objectives

Sialidase is a key enzyme for enzymatic analyses of sialylo-compounds (Sugiura et al., 1980) and a candidate therapy for spinal cord injury (Mountney et al., 2010). The *Micromonospora viridifaciens* sialidase is the best characterized bacterial sialidase that has a broad substrate specificity hydrolysing α -2,3 , α -2,6 and α -2,8 glycosidic linkages (Aisaka et al., 1991). This study aimed to improve hydrolase activity of *M. viridifaciens* sialidase by protein engineering.

It has been evidenced by many examples of directed evolution of enzymes that promiscuous activity can be substantially improved with one or few mutations, often with a relatively low effect on the native activity (Khersonsky et al., 2006). A diminish return exists in evolution that the closer the process is to the optimum, the smaller the benefit is achieved by mutations (Tokuriki et al., 2012), indicating that improvement of natural function would be more difficult and less substantial than improving side activity. Moreover, a trade-off between stability and activity has been commonly observed in protein engineering, *i.e.*, most mutations that contribute to improving catalytic proficiency have been shown destabilizing (Tokuriki et al., 2008). In this study, 20 amino acids in or near the active site were targeted for site-saturation mutagenesis. Mutant libraries were tested on both a non-natural substrate (pNP-Neu5Ac) and a natural substrate (cGMP) in order to investigate if and how the potential for optimizing the enzyme towards non-natural substrates (side activity) is higher than for natural substrates (native activity). We further examined whether improvement of catalytic proficiency would correlate with decreased stability introduced in mutated sites.

3.2 Experimental considerations

Twenty amino acids within 10 Å of the sialic acid binding site (10 sites comprising part of the sialic acid binding pocket, three sites in close proximity to the catalytic Tyr, seven surface-exposed amino acids that possibly affect the interaction with the sialyl-substrate) were selected for mutagenesis.

In order to facilitate the screening in microtiter plate format, *B. subtilis* SCK6 that has high transformation efficiency was used as a production host for secretory expression of the wild type sialidase gene as well as mutant libraries. The screening method comprising enzyme expression

and sialidase assay in 96-well plate format was validated by use of the wild-type gene. Sialidase assay was performed using the artificial substrate pNP-Neu5Ac or the natural substrate cGMP, respectively. For each library, screening was done with 90 transformants and six wild type enzymes per plate. Activities of mutant enzymes were normalized according to those of the wild type enzymes.

When tested on pNP-Neu5Ac, the increase in the sialidase activity displayed by the most active mutants was substantially higher than on cGMP. The mutants exhibiting the largest increased activity towards pNP-Neu5Ac were sequenced and the corresponding proteins were purified. To verify the screening results their specific activities were tested on pNP-Neu5Ac. In addition, these proteins were assayed against three more natural substrates 3'-sialyllactose, 6'-sialyllactose and colominic acid to confirm the generality of the observation that none mutants showed significant improvement on cGMP.

The stability change of $\Delta\Delta G$, a measure of potential chemical variation was computed by PoPMuSiC for each amino acid. The correlation between computed $\Delta\Delta G$ and activity of mutants gives a site-specific estimate of the evolvability, *i.e.*, a high value for a site suggests that most mutations in the site are unlikely to occur in natural evolution and are associated with major chemical changes and decreased stability.

3.3 Main results and conclusion

The number of mutants displaying increased sialidase activity on pNP-Neu5Ac was considerably higher than on cGMP. Moreover, the most active mutants displayed more than 10-fold increase in activity on pNP-Neu5Ac, while no mutants had >160 % of the wild-type-level activity when screened on cGMP.

The enzymes corresponding to the most active mutants towards pNP-Neu5Ac in the screen were purified and the screening result was confirmed by comparing the specific activities of these mutant enzymes with that of wild type enzyme. Specifically, the most proficient mutant displayed the highest specific activity on pNP-Neu5Ac with a 27-fold increase. This mutant possessed double mutations of I91E and S201L, however, the activity increase was mostly attributed to the mutation of S201L. The largest increases were observed with amino acids (V148, F155, L170, F203 and S201L) located in the sialic acid-binding pocket in close vicinity to the *N*-acetyl group of sialic acid.

When tested on natural substrates 3'-sialyllactose, 6'-sialyllactose and colominic acid, none of these purified enzymes showed significant activity increase, corroborating the observation from the screening on cGMP.

No correlation was found between activity on cGMP and the average PoPMuSiC $\Delta\Delta G$ value, implying that there is little room for activity increase towards cGMP (*i.e.*, this protein is near the proficiency optimum on the natural substrate). On the contrary, there was a significant correlation between the increased activity on pNP-Neu5Ac and the predicted $\Delta\Delta G$ value, suggesting that there is much more room for activity increase towards the artificial substrate and this improvement correlates with chemical changes and decreased stability introduced in the mutated sites.

Mutants of *Micromonospora viridifaciens* sialidase have highly variable activities on natural and non-natural substrates

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Submitted to Protein Engineering, Design and Selection

Abstract

This study aimed to improve the hydrolase activity of the well-characterised bacterial sialidase from *Micromonospora viridifaciens*. The enzyme and its mutants were produced in *Bacillus subtilis* and secreted to the growth medium. 20 amino acid positions in or near the active site were subjected to site-saturation mutagenesis and evaluated on the artificial sialidase substrate 2-*O*-(*p*-nitrophenyl)- α -*D*-*N*-acetylneuraminic acid and on the natural substrate casein glycomacropetide. A considerably higher fraction of the mutants exhibited increased activity on the artificial substrate compared to the natural one, with the most proficient mutant showing 27-fold improvement. In contrast, no mutants displayed more than a 2-fold increase in activity on the natural substrate. To gain further insight to this important discrepancy, we analysed the stability of mutants using the PoPMuSiC software, a property that also correlates with the potential for introducing chemical variation. We found a significant correlation between improved hydrolase activity on the artificial substrate and reduced apparent stability. Together with the minor improvement on the natural substrate this shows an important difference between naturally evolved functionality and new laboratory functionality. Our results suggest that when engineering sialidases and potentially other proteins towards non-natural substrates that are not optimized by natural evolution, major changes in chemical properties are advantageous, and these changes tend to correlate with decreased stability, partly explaining commonly observed trade-offs between stability and proficiency.

Keywords

Micromonospora viridifaciens/mutagenesis/protein evolution/sialidase/stability-activity trade-off

Introduction

In protein evolution an important characteristic of enzymes (and other proteins) is their ability to evolve to adapt to new functions (Tokuriki and Tawfik, 2009). This encompasses both altered substrate specificity (but same reaction type) as well as catalysis of new reactions. In fact, it is clear that many proteins besides their main activity also can catalyse other reactions, normally with lower efficiency. These enzymes appear to have arisen from generalist ancestors after adaptation towards high specificity and activity for a given reaction (Khersonsky and Tawfik, 2010).

To obtain and maintain functionality, a protein needs to fixate specific amino acids at important positions in the structure. These functionally important residues have been shown to generally reduce protein stability (Tokuriki et al., 2008). Enzyme stability is normally not

maintained at a level substantially higher (typically 5–15 kcal/mol) than that required to function in its natural setting (Branden and Tooze, 1999). Trade-offs between stability and activity are a major issue in protein engineering, and several causes for these trade-offs have been suggested (Tokuriki et al., 2008; Kepp and Dasmeh, 2014). As adaptation towards new functionality often proceeds via destabilizing mutations, an evolution trajectory appears to go via initial accumulation of stabilizing mutations to allow incorporation of functional often destabilizing mutations (Tokuriki et al., 2008). This trade-off between stability and activity has been observed both in laboratory and natural evolution (Tokuriki et al., 2012; Nagatani et al., 2007; Studer et al., 2014).

Laboratory evolution of enzymes has demonstrated that in many cases latent promiscuous activities can be improved with a minimal amount of mutations, often with a relatively low effect on the main activity (Khersonsky et al., 2006). When approaching the optimum, a diminishing return is seen, that is, additional mutations will lead to relatively smaller improvement in proficiency (Tokuriki et al., 2012). This would indicate that improvement of an enzyme with respect to its natural function (for which it has been specialized for) would be more difficult and less substantial than improving side activities. However, it should be noted that many natural enzymes appear to be far from their proficiency optimum because of additional constraints on the proteins (Tokuriki et al., 2012).

In laboratory evolution of enzymes, the construction of smaller libraries is beneficial as this will reduce the amount of screening needed. To do so, semi-rational mutant designs in which select residues are subjected to site-saturation mutagenesis can be useful. As the observations on stability-function trade-off indicates that most functional mutations are destabilizing, this might be a parameter useful for selection of residues for mutagenesis given that the target enzyme is stable enough to accommodate them.

In this study a sialidase (EC 3.2.1.18) from *Micromonospora viridifaciens* was used as a model enzyme. Sialidases are enzymes that hydrolyze sialic acids or sialyl groups of glycans and are found in all domains of life. Bacterial sialidases are classified in glycosyl hydrolase family 33 and their structures are of the six-bladed β -propeller type (Crennell et al., 1993). As retaining glycosidases, they employ a double-displacement mechanism and use a Tyr residue as catalytic nucleophile (Watts et al., 2003) while a conserved Asp-Glu pair is involved in general acid/base catalysis (Chan et al., 2012). Interestingly, this enzyme can retain function upon mutation of either of the catalytic residues (D92, E260, and Y370) (Watson et al., 2003; Chan et al., 2012). A conserved arginine triad (R68, R276, and R342) stabilizes the carboxyl group of sialic acid

(Crennell et al., 1993). The *M. viridifaciens* sialidase is the best characterized bacterial sialidase. It has a broad substrate specificity hydrolysing both α -2,3-, α -2,6- and α -2,8-glycosidic linkages and exhibits maximum activity at pH 5 (Aisaka et al., 1991). The k_{cat}/K_m of the enzyme is $8.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for hydrolysis of the artificial substrate 2-*O*-(*p*-nitrophenyl)- α -*D*-*N*-acetylneuraminic acid (pNP-Neu5Ac) and $0.36 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for hydrolysis of the natural substrate 3'-sialyllactose (Watson et al., 2003).

The application of sialidases has gained significant interest for the synthesis of sialoglycans using their trans-sialidase activity (Schmidt et al., 2000; Jers et al., 2014). Several studies have demonstrated potential uses of sialidases as therapeutics targeting sialoglycans, most notably in case of nervous system injuries. Upon injury, inhibitors of axon regeneration some of which bind to sialylated receptors, accumulate to halt axon outgrowth. In rats, the administration of sialidase improved spinal axon outgrowth and recovery after spinal cord contusive injury (Yang et al., 2006; Mountney et al., 2010). While several protein-engineering efforts have been directed towards improving trans-sialidase activity (Paris et al., 2005; Pierdominici-Sottile et al., 2014; Jers et al., 2014), to our knowledge no attempts have been made to improve hydrolase activity of sialidases despite their importance in disease and inflammation. With potential applications in clinical settings emerging, we set out to probe the potential for improving the hydrolase activity of the well-characterized sialidase from *M. viridifaciens* by mutagenesis.

In this study, we subjected select active site-amino acids to site saturation mutagenesis and tested the mutant libraries on both a natural substrate casein glycomacropeptide (cGMP) containing sialic acid α -2,3-linked to galactose and α -2,6-linked to *N*-acetylgalactosamine (Saito and Itoh, 1992), but also on the widely used non-natural test substrate pNP-Neu5Ac. This substrate differs from its natural counterparts by having Neu5Ac linked to a *para*-nitrophenyl group opposed to most commonly galactose in natural glycans. When using pNP-Neu5Ac, a high proportion of mutants displayed activities substantially higher than the wild type and overall up to 25-fold higher activity. In contrast, screening on the natural substrate cGMP led to much less frequent and limited improvements. The improved activities on pNP-Neu5Ac correlated with increased chemical variation and computed stability changes, confirming the hypothesis that the natural substrates resemble an evolved proficiency optimum, whereas activity on the non-natural substrate is associated with major adaptation upon variation in chemical properties. Thus, we conclude that for optimizing a protein towards non-natural substrates not optimized by natural evolution, major changes in chemical properties that tend to correlate with decreased stability are more likely to

successfully improve functionality, consistent with a trade-off between stability and proficiency as widely debated in protein engineering (Tokuriki et al., 2008; Dellus-Gur et al., 2013).

Materials and methods

Substrates

pNP-Neu5Ac and colominic acid were purchased from Sigma-Aldrich (Steinheim, Germany). 3'-sialyllactose and 6'-sialyllactose were obtained from Carbosynth (Compton, United Kingdom). The commercial cGMP product LACPRODAN CGMP-20 with a sialic acid content of about 9 % (w/w) was supplied by Arla Foods (Viby, Denmark).

Mutant selection and bioinformatics analysis

Pymol v1.3 (Schrödinger) was used to identify amino acids within 10 Å of the sialic acid binding site, based on the crystal structure with PDB code 1EUS (Gaskell et al., 1995). Individual sites were selected based on manual inspection of the structure. Since the number of mutations prevented experimental stability assessment, we analysed the stabilities of the mutants using the PoPMuSiC software (Dehouck et al., 2011) with the structure templates PDB 1EUS, 1EUT, 1EUU and 1EUR. This method has been shown previously to give good accuracy for batches of mutants in several studies (Worth et al., 2011; Kepp, 2014). PoPMuSiC computes the $\Delta\Delta G$ (change in free energy of protein folding) for a mutant based on parameterized, environment-dependent empirical substitution frequencies in orthologs, i.e. it measures the propensity of a given substitution based on empirical substitution patterns. Rare substitutions are thus associated with large variations in chemical properties that are likely to change the protein function more, and these have resulting larger $\Delta\Delta G$, i.e. are considered destabilizing.

Bacterial strains and growth conditions

Escherichia coli NM522 was used for plasmid propagation. *E. coli* was grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) shaking at 37 °C. *Bacillus subtilis* strains SCK6 (Zhang and Zhang, 2011) and WB800 (Wu et al., 2002) were used for protein synthesis. *B. subtilis* was grown shaking at 37 °C in LB medium and C-medium (Martin-Verstraete et al., 1990) supplemented with 5 g/L glucose and 2 % LB medium (CG-LB medium). The growth media were supplemented with kanamycin at 25 µg/mL for *E. coli* and 50 µg/mL for *B. subtilis*.

DNA manipulation and strain construction

The protein sequence of *M. viridifaciens* sialidase (Uniprot ID Q02834) contains a signal sequence constituted by amino acids 1-37 (Sakurada et al., 1992). A synthetic gene encoding the mature protein (38-647) initiated by methionine and with a C-terminal 6xHis-tag was codon optimized for expression in *B. subtilis* and synthesized by DNA2.0 (Menlo Park, CA, USA). The gene fragment was inserted in the vector pDP66K-PME (Øbro et al., 2009) between the *NcoI* and *HindIII* restriction sites replacing the PME-encoding gene and placing the sialidase gene in frame with the CGTase signal peptide and under control of the P32 promoter (Penninga et al., 1996). The use of the *NcoI* restriction site, led to mutation of I38 to glycine. Amino acid numbering will follow that of the wild type protein. Site saturated mutagenesis of 20 positions was done by GeneArt (Regensburg, Germany). Two point mutations, I91E and S201L, were introduced in the wild type gene by overlapping primer mutagenesis using the oligonucleotides displayed in Table 1. The PCR products were restricted with *NcoI* and *HindIII* and inserted in pDP66K-PME. The 20 libraries of plasmids and the two constructed single mutant-encoding plasmids were used to transform *B. subtilis* SCK6 (Zhang and Zhang, 2011). This strain contains the *comK* gene under control of a xylose-inducible promoter and was transformed with plasmid DNA as described previously (Silva et al., 2013). *B. subtilis* WB800 was transformed with pDP66K containing the gene encoding the wild type sialidase using a one-step transformation protocol (Jarmer et al., 2002).

Table 1. List of oligos. Mutated codons are shown in bold. MCS is the multiple cloning site.

Name	Sequence	Description
pDP66K fwd	CGAATTCGGTCTCTCGGGATATGATAAG	Anneals upstream MCS
pDP66K rev	GCCTTCTGCGTCCGGTCGATCAGGGAGGATATCG	Anneals downstream MCS
Mv I91E Fwd	CCCAACAGGAG AA GATGCACCTGGACCGAACTC	I91E
Mv I91E Rev	CAGGTGCATCT TT CTCCTGTTGGGCGGCCGTC	I91E
Mv S201L Fwd	TGGATGGCGCT TT GAGATTGCTGCTAGCGGTGAAGG	S203L
Mv S201L Rev	CAGCAAATCT CA AGCGCCATCCAGGATCCGG	S203L

Screening of site-saturation libraries

For each of the site-saturation libraries, screening was done in 96-well microtiter plate format with 90 transformants (4.5 fold coverage) and six wild type enzymes per plate. *B. subtilis* was transformed with the site-saturation library and wild type plasmid, and after overnight incubation at 37 °C, the transformants were transferred to 125 µL LB medium with kanamycin in a 250 µL round bottom microtiter plate (Nunc), covered with Airpore sheet (Qiagen) and incubated at 37 °C with

shaking at 250 rpm for 6 hours. The preculture step reduced well-to-well variation from growth. From this preculture, 5 μ L was used to inoculate 500 μ L CG-LB medium with kanamycin in a 2 mL deepwell plate (Eppendorf). The plate was covered with airpore sheet and incubated at 37 °C with shaking at 250 rpm for 16 hours. At this point, the supernatants containing the secreted enzymes were harvested by centrifugation for 15 min at 5000 g and 4 °C and kept on ice or stored at -20 °C in 10 % glycerol until used in the assays.

Protein production

For larger scale production of selected enzyme variants, 50 mL cultures were grown by inoculating 50 mL LB medium with kanamycin to OD₆₀₀ of 0.05 and incubation with shaking at 37 °C for 23 hours. At this point, the supernatant was harvested by centrifugation for 15 min at 5000 g and 4 °C and passed through first a 0.50 and then a 0.20 μ m filter. The ~48 mL supernatant was then concentrated to less than 1 mL using a Vivaspin20 10 MWCO concentrator (Sartorius AG), mixed with 10 mL purification buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) with 10 mM imidazole and concentrated to 1.5 mL. The concentrated sample was applied to a His spin trap column (GE Healthcare) and washed once with 600 μ L of the same buffer, then four times with 600 μ L purification buffer containing 30 mM imidazole and finally eluted in 400 μ L purification buffer with 300 mM imidazole. To reduce imidazole concentration to less than 0.5 mM, several rounds of concentration and dilution in storage buffer (20 mM sodium phosphate, 100 mM NaCl and 10 % glycerol, pH 7.4) were done using Vivaspin6 10 MWCO concentrator (Sartorius AG). Final volume was adjusted to 500 μ L. Protein concentration was estimated using the BCA assay (Thermo Scientific) with BSA as standard.

Sialidase activity assays

Two different enzyme assays were used for testing sialidase activity: one using the substrate pNP-Neu5Ac and another for testing activity on various natural substrates. The pNP-Neu5Ac hydrolase assay was done in a 50 mM phosphate-citrate buffer at pH 7 with 0.75 mM pNP-Neu5Ac at 30 °C using various amounts of enzyme. In the screen, 5 μ L of culture supernatant was used in a 100 μ L reaction. Reactions were started by addition of substrate and monitored continuously in a spectrophotometer at 410 nm absorbance. Reactions were followed for 1 h, and initial reaction rates were calculated as the slope of the linear portion of the curves and presented in the following as percent of the wild type enzyme activity. For testing the purified enzymes, 1.5 μ g/mL His-tagged

wild type enzyme was used, while for the mutant enzymes a 5-14 times lower concentration was used. For measurement of hydrolysis of natural substrates, reactions were performed with either 1 mM 3'-SL, 1 mM 6'-SL, or 1 mM cGMP-bound sialic acid in 50 mM phosphate-citrate buffer (pH 5 or 7) using 10 μ L of 25-fold diluted culture supernatant in 50 μ L reactions or 5 μ g/mL purified enzyme. Reactions were started by addition of enzyme and stopped by adding H₂SO₄ to a final concentration of 45 mM. Quantification of free sialic acid was done using a modified thiobarbituric acid assay (Jers et al., 2014).

Results

Cloning, expression and purification of *M. viridifaciens* sialidase in *B. subtilis*

The sialidase from *M. viridifaciens* was previously produced in bacterial hosts *Streptomyces lividans* and *E. coli* (Sakurada et al., 1992; Watson et al., 2003). In order to allow efficient secretion to the culture medium, we attempted to use *B. subtilis* as a production host since this would simplify screening in microtiter plate format. Expression of a codon-optimised gene in *B. subtilis* SCK6 (a strain devoid in two of the major secreted proteases) allowed production and secretion of the 6xHis-tagged sialidase (Figure 1). Besides the full length protein, a smaller form of ~28 kDa was observed similar to the size of the C-terminal, non-catalytic form reported previously (Sakurada et al., 1992). It was attempted to produce the enzyme in *B. subtilis* WB800, a strain knocked out in eight proteases, but this did not reduce degradation (Figure 1) and hence the strain *B. subtilis* SCK6 was used for the reported work.

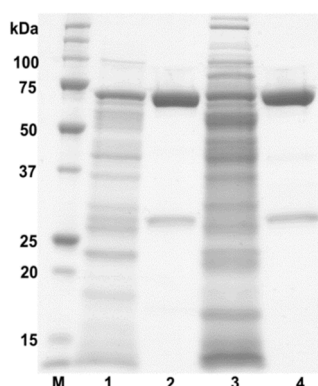


Fig. 1. SDS-polyacrylamide gel of *M. viridifaciens* sialidase produced in *B. subtilis*. M is a molecular weight marker, Lanes 1 and 2 show supernatant and purified sialidase produced in *B. subtilis* strains SCK6 and lanes 3 and 4 show supernatant and purified sialidase produced in *B. subtilis* WB800.

Inspection of active site amino acids

In order to investigate the possibility of improving the catalytic proficiency of sialidases, we mutated a number of positions in or near the active site. To maximize the possibility of identifying relevant mutations, we performed single-amino acid site-saturation mutagenesis in each of 20 selected positions. Positions were selected based on inspection of the solved structure (Gaskell et al., 1995; 1EUS). These included nine sites comprising part of the sialic acid binding pocket: I69, A93, S130, D131, P132, V148, F155, L170, F203, and D259. Of these, D131 and D259 form hydrogen bonds with the inhibitor DANA in the crystal structure and P132 is buried underneath the pocket. Three amino acids N310, N311, and S369 are located close to the catalytic tyrosine, and we envisioned that they might modulate the covalent intermediate or transition state and thus affect turnover. A number of surface-exposed amino acids were selected because they might change the interaction with the substrate molecules to which sialic acid is bound. These included G90, I91, G154, Q153, A156, and F234. Finally, N66 a second-sphere, surface-exposed residue in close vicinity to conserved R68 was selected. None of the active site amino acids strictly conserved in all sialidases (R68, D92, E260, R276, R342, and Y370) were targeted. The sites selected for mutagenesis are presented in Figure 2.

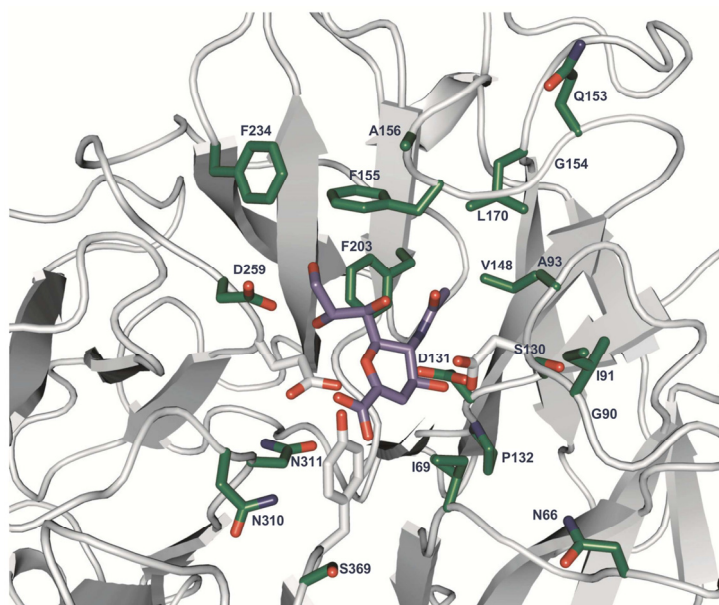


Fig. 2. Amino acid positions in the active site selected for mutagenesis. Selected amino acids are shown in green, catalytic residues D92, E260, and Y370 are in grey while the structure-bound ligand is in blue.

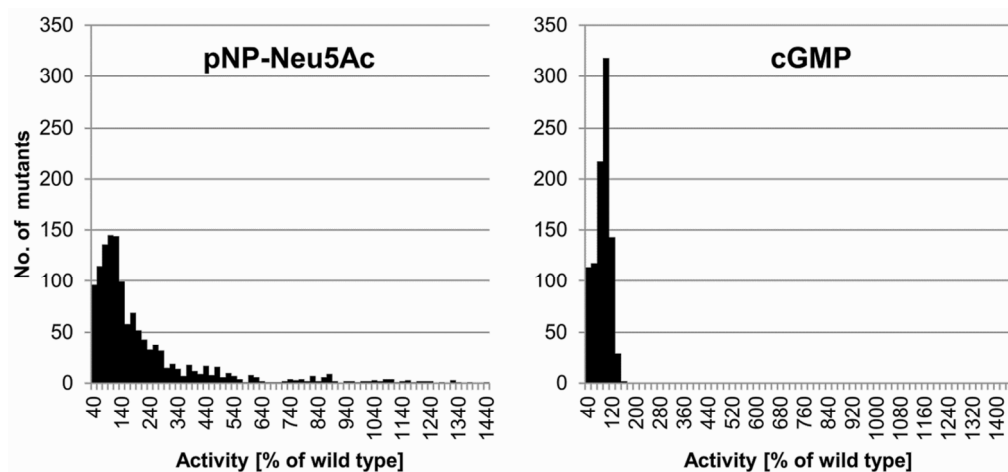


Fig. 3. Screening of mutant libraries. Histogram presenting the activity (% compared to wild type) of 1800 mutants tested on (A) pNP-Neu5Ac and (B) cGMP with a bin size of 20 %. 475 and 859 mutants showed no or low activity (0-20 % wild type activity) on pNP-Neu5Ac and cGMP respectively and are not shown in the histograms.

Screening of site saturation libraries reveals variable activities on pNP-Neu5Ac and cGMP

The mutant libraries were synthesized to contain genes encoding all 20 amino acids in a given position. The DNA was used to transform *B. subtilis* and transformants were picked to a 96-well microtiter plate. In initial experiments, a relatively high well-to-well variation was observed due to differences in inoculum used. That was alleviated by synchronizing growth by pre-culturing the cells to early stationary phase, and using this culture for inoculating the enzyme production plate. Cells were removed by centrifugation and supernatant containing the secreted enzyme was assayed for enzyme activity on the substrates pNP-Neu5Ac and cGMP. To validate the method, this pipeline was tested using DNA encoding the wild type enzyme. This was repeated three times, and the activities of individual wells were between 80-125 % of the average activity when tested on pNP-Neu5Ac. Next, the libraries were tested on the substrates pNP-Neu5Ac and cGMP. For each amino acid 90 transformants were tested and activities were normalized with respect to the activity of the wild type enzyme. The pooled results for all transformants tested are presented in a histogram (Figure 3). Results for individual positions are presented in Figure S1. When screened on cGMP, a larger number of mutants showed no or low activity than on pNP-Neu5Ac. Further, the majority of active mutants exhibited wild type-level activity on cGMP and very few mutants had an increased activity on this substrate. No mutants had an activity above 160 %. When tested on the artificial

substrate pNP-Neu5Ac, the majority of mutants also exhibited wild type-level activity but the number of mutants exhibiting increased activity was considerably higher, and the most active mutants displayed more than 10-fold increase in activity under the conditions used.

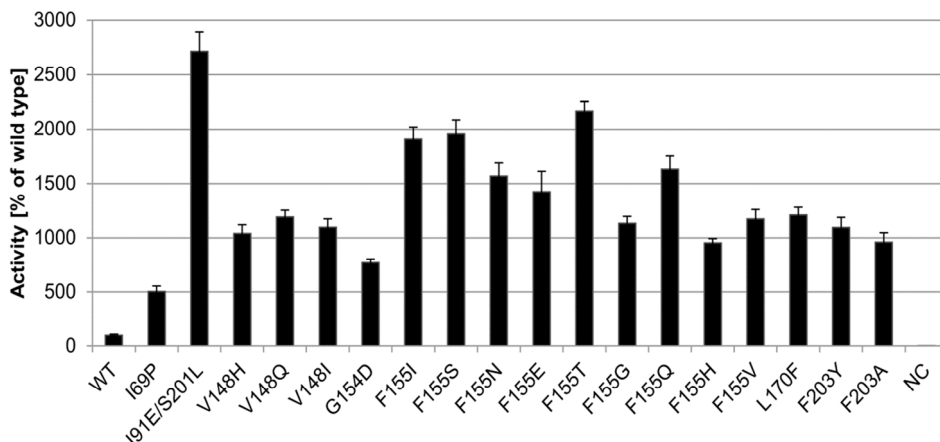


Fig. 4. Specific activity of the most proficient mutants selected on pNP-Neu5Ac. Activity increments compared to activity of the wild type sialidase. Negative control (NC) was a reaction without enzyme added. Average of four replicates.

Mutations improving the hydrolysis activity on pNP-Neu5Ac

To provide a foundation for interpreting the reasons underlying the difference in improvements observed, we sequenced the variants that showed the largest increase in activity towards pNP-Neu5Ac (Table S1). Further, we wanted to verify the screening results on a set of the variants exhibiting the largest increase in activity on pNP-Neu5Ac. To this end, plasmids were purified and entire genes were sequenced and the corresponding strains were grown in larger scale to allow production and purification of the 6xHis-tagged enzymes. We found that the most proficient mutant in the screen possessed both mutation I91E but also S201L. The corresponding enzymes were produced and tested on pNP-Neu5Ac (Figures S2 and 4). This largely confirmed the screening results except it appeared that the specific activities of most improved variants were underestimated in the screen, and mutants with more than 20-fold improvement in activity was observed. The mutant I91E/S201L exhibited the highest activity with a 27-fold improvement. When testing the individual single mutants, I91E had wild-type like activity whereas S201L could explain most of the improvement (Figure S3).

As indicated in the screen, neither of the mutants showed significant improvement on the natural substrate cGMP. To confirm the generality of this observation, we tested the purified enzymes on three other natural substrates 3'- and 6'-sialyllactose (Neu5Ac α -2,3-, and α -2,6-linked to galactose respectively), and colominic acid (Neu5Ac polymer linked by α -2,8 bonds) (Figure S4). The results confirmed the overall observation, with some variants showing slight improvements and others showing significantly reduced activity on these substrates. To allow detection of the chromophore pNP in a continuous assay, the screening using pNP-Neu5Ac was done at pH 7, while the cGMP screen was done at pH 5, the optimum pH for the wild type enzyme (Aisaka et al., 1991). To assess whether this discrepancy could account for the differences observed between the two substrates, we analysed the purified mutant enzymes on 3'-sialyllactose at pH 5 and 7 (Figure S4). This confirmed that the observed improvements were not due to a change in pH optimum or range.

Structure analysis and hypothesis for effect of mutations

After having showed that the mutagenesis strategy preferentially yielded mutants with improved activity on pNP-Neu5Ac, we investigated whether the number of improving mutations would correlate with the chemical variation and stability effects in the library. To this end, we used PoPMuSiC where the computed stability change $\Delta\Delta G$ for each mutation provides a measure of the environment-dependent substitution propensity based on empirical substitution frequencies of orthologs, and these can then be summed for each of the 20 sites. A large positive value of $\Delta\Delta G$ thus implies both a highly destabilizing mutant and a natural-evolution-wise unlikely substitution.

We then assessed whether there were correlations between these $\Delta\Delta G$ values for all 20 individual sites and the number of mutants with higher, similar or lower activity compared to the wild type enzyme. This approach gives a site-specific estimate of the evolvability and a high value for a site implies that most mutations in the site are unlikely to occur in natural evolution and are associated with loss of stability due to major changes in the chemical properties of the protein. We found no correlations between activity on cGMP and the PoPMuSiC results, indicating that the protein is nearly optimal (by evolution) for activity on natural substrates, and any changes in the sites are likely to have small or negative effect on activity irrespective of chemical properties and stability (Figure S5).

In contrast, there was a markedly different and statistically significant correlation between the average PoPMuSiC $\Delta\Delta G$ of a site and the site-specific activities of the most active mutants on the non-natural substrate pNP-Neu5Ac. This indicates much more room for improvement, and

importantly, improved activity is associated with sites that exhibit major chemical changes and decreased stability (Figure 5).

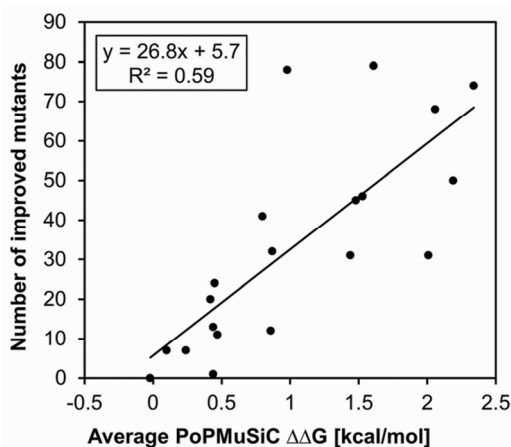


Fig. 5. Correlation between potential for chemical variation and improvement towards hydrolysis of pNP-Neu5Ac. PoPMuSiC predictions were used as a measure of potential chemical variation and was for each amino acid position plotted against the number of mutants that exhibited improved activity towards pNP-Neu5Ac.

We finally considered the amino acid positions that yielded the largest increases in activity on pNP-Neu5Ac. For the most improved variants, the mutations in V148, F155, L170, and F203 were all located in the sialic acid-binding pocket within 4–5 Å of the *N*-acetyl group of sialic acid (Figure 6). The mutation S201L that was functionally important in the most improved variant was located in the second sphere in close vicinity to these four sites. Sequencing of mutants of the two aspartic acid residues involved in sialic acid binding indicated that D131 only remained active when mutated to glutamic acid. However, this reduced the activity on pNP-Neu5Ac, and no activity was observed on cGMP, indicating its functional importance. For D259, mutation to glutamic acid increased activity towards pNP-Neu5Ac while other mutations led to reduced activity and in this case, we found no mutants active on cGMP.

Discussion

In this work, we aimed to assess the potential for improvement of a well-characterized sialidase towards both artificial and natural substrates. To this end, we designed, cloned, expressed, and characterized mutants resulting from saturated mutagenesis at 20 positions near the active site.

Positions were selected based on inspection of the solved structure with PDB code 1EUS (Gaskell et al., 1995). None of the strict catalytic residues (R68, D92, E260, R276, R342, and Y370) were selected, but except from that, conservation within the sialidase family was not considered since we wanted to optimize *in vitro* proficiency, and these conserved sites are likely to add additional constraints on the protein relating to e.g. *in vivo* regulation and modification.

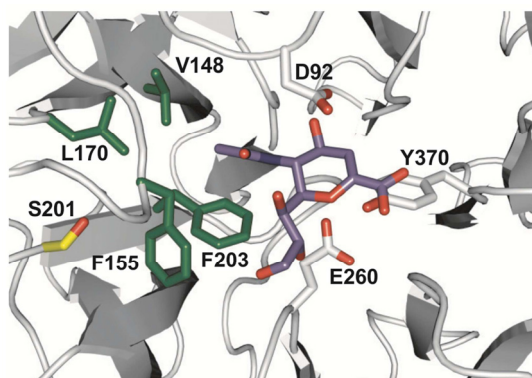


Fig. 6. Representation of the positions with most improved variants. The mutated positions V148, F155, L170, and F203 are shown in green, the site S201 that yielded the most improving mutation is shown in yellow while the catalytic residues D92, E260, and Y370 are shown in grey. The ligand is shown in blue.

The potential for improving the catalytic efficiency of an enzyme largely depends on how well it is optimized for its particular function. In a previous study the catalytic efficiency $k_{\text{cat}}/K_{\text{m}}$ was determined for the substrate pNP-Neu5Ac and a natural substrate 3'-sialyllactose indicating a 10-fold higher efficiency of the enzyme towards the pNP-substrate (Watson et al., 2003). For other classes of enzymes, large rate accelerations have been observed when using artificial pNP-substrates (Indurugalla et al., 2006), reflecting elevated intrinsic reactivity of the substrate rather than the enzyme being optimized for this particular substrate. Consequently, we wanted to investigate if and how the potential for optimizing the enzyme towards non-natural substrates is higher than for natural substrates. To test this, we assayed the mutant libraries on two substrates, the artificial test substrate pNP-Neu5Ac and the natural substrate cGMP derived from casein. This demonstrated a much higher proportion of beneficial mutations with respect to hydrolysis of the artificial substrate compared to the natural one and the best mutant (I91E/S201L) had a 27-fold improvement in activity whereas for cGMP only slight improvement was observed. This reflects substrate specific differences and consequently mutations selected specifically to favor action on the

artificial substrate. This is in line with observations on promiscuous enzymes, where it is well-established that while changing main function can be difficult, a weaker side activity often can be substantially improved with one or few mutations often without significantly affecting the main activity (Khersonsky et al., 2006).

Both in laboratory and natural evolution there is a trade-off between stability and function (Tokuriki et al., 2012; Nagatani et al., 2007; Studer et al., 2014). Since we in this study analysed single mutants only, such effects would not be readily apparent. Destabilizing mutations would be expected to be tolerated by our model enzyme, since it appears reasonably stable (temperature optimum at 52 °C) (Watson et al., 2003). When analyzing the stability change of mutations, there was a significant correlation between the average PoPMuSiC $\Delta\Delta G$ and activity on the artificial substrate for the most improved mutants while no correlation with activity on the natural substrate was observed. This would be consistent with the view that the protein is nearly optimal (by evolution) for activity on natural substrates: Any changes are then likely to have small or negative effect on activity regardless of chemical properties and stability, i.e. we have probed a proficiency optimum (in evolutionary terms, resembling a partial fitness optimum, if other selection pressures on the protein are ignored).

In contrast, when optimizing a protein for a new, non-natural substrate, there is much more room for improvement since the substrate is no longer optimal, and importantly, improved activity is associated with sites that exhibit major chemical changes and thus, decreased stability. The observation that the activity towards non-native substrates can be readily improved and that the improvement correlates with the potential for introducing chemical variation in a given amino acid position has direct implications in protein engineering. On the other hand, none of the single mutants were markedly improved towards the natural substrate. In nature, the enzyme is likely to have been selected for efficient hydrolysis of galactose-linked sialic acid and could potentially, in sequence space, have reached a local optimum. In that case, it might require multi-mutants to enable significant improvements in activity which would not be captured by our strategy (Tracewell and Arnold, 2009).

When analyzing the effect in individual positions, the functional importance of especially D141 and D259 that interact directly with sialic acid, was apparent with most mutants showing no activity. For reaction on pNP-Neu5Ac, mutation to the chemically similar but larger amino acid glutamic acid was tolerated (2-fold reduction and 2.5-fold increase in activity respectively). On cGMP, both mutants had no detectable activity indicating a difference in the position of sialic acid

in the binding pocket. Further, it appeared that the largest improvements were observed with amino acids (V148, F155, L170, F203 and S201L) located in the sialic acid-binding pocket in close vicinity to the *N*-acetyl group of sialic acid. This might indicate that positioning of sialic acid in the binding pocket was changed likely due to secondary effects of the nitrophenyl group. This would imply that the major functional role of the mutations would be to allow an alternative positioning of sialic acid that would lead to a more optimal positioning of the nitrophenyl group. This in turn would not affect the overall catalytic activity of the enzyme as was also observed for the cGMP data set.

pNP-Neu5Ac is one of the most frequently used test substrates for sialidases. While the use of artificial test substrates allows easy and fast screening, it is not necessarily indicative of the activity towards the natural substrate (Lee, 2010). This is also apparent in this data set, namely that there was not a clear correlation between activity of mutants on the artificial and natural substrate. The use of pNP-Neu5Ac as an indicator of potential sialidase activity may be reasonable, but with respect to characterisation of biological functions of sialidases, more relevant, natural substrate should be chosen.

In conclusion, using a well-studied sialidase as an example, we used site-saturation mutagenesis of 20 sites close to the active site and subsequent activity characterization of mutants to investigate the ability of the enzyme to improve activity towards both non-natural and natural substrates. We show that while there is little room for improvement towards a natural substrate, consistent with the protein being near the proficiency optimum, there is substantial room for producing highly proficient sialidases with activity towards pNP-Neu5Ac. Furthermore, this tendency of improvement correlated with chemical variation introduced in the mutated sites, which again correlates with stability decrease, as computed by the PoPMuSiC algorithm. This study thus provides a mechanistic explanation for stability-activity trade-offs found in protein engineering when the investigated activities are not towards the natural substrate, which is commonly the case.

Acknowledgements

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Supplementary material

Mutants of *Micromonospora viridifaciens* sialidase have highly variable activities on natural and non-natural substrates

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This supplement contains:

Table S1. Overview of sequenced mutants with increased activity on pNP-Neu5Ac

Figure S1. Histogram presenting the activity of screened mutants in individual positions.

Figure S2. SDS-polyacrylamide gel of 6xHis-tagged mutant enzymes

Figure S3: Activity of selected mutant I91E/S201L and the single mutants I91E and S201L

Figure S4. Specific activity of mutants on natural substrates

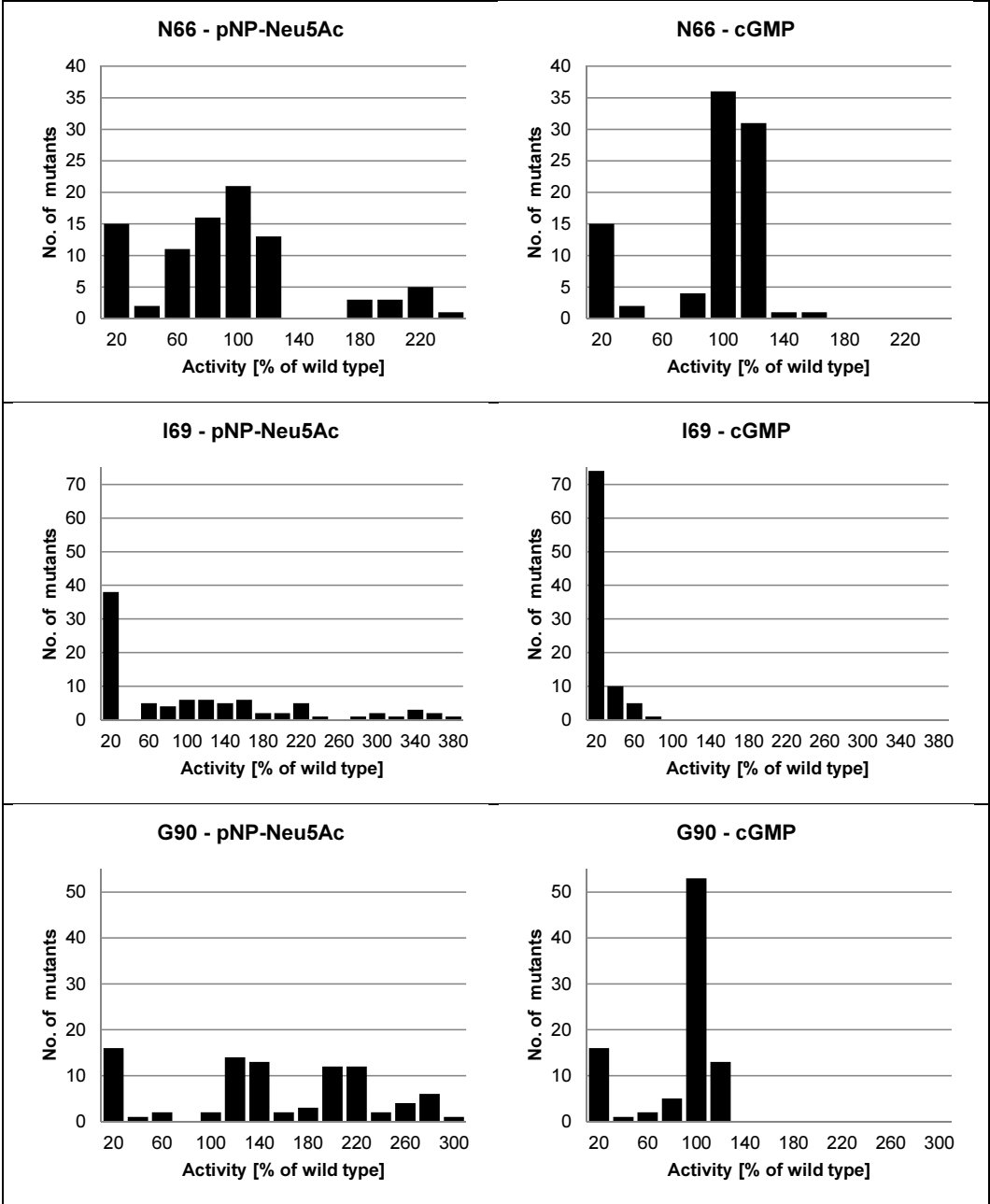
Figure S5. Correlation between PoPMuSiC predictions and activity of mutants

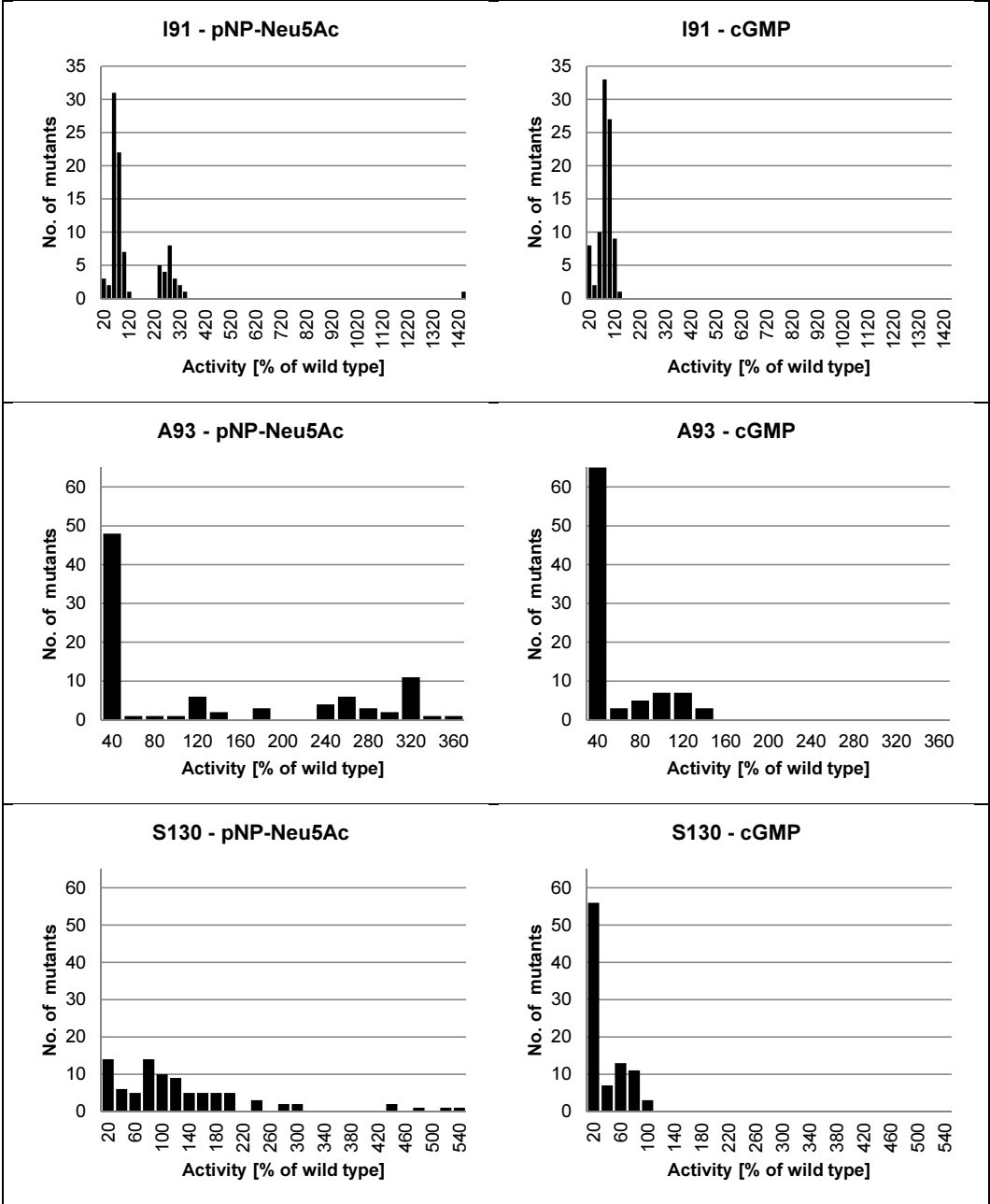
Table S1. Overview of sequenced mutants with increased activity on pNP-Neu5Ac. Represented are the introduced mutation (Mut), number of clones (n), and activity in the screen on pNP-Neu5Ac and cGMP. When more than one clone contained the mutation, activity is given as the average. Mutants purified for further validation of screen are marked with bold.

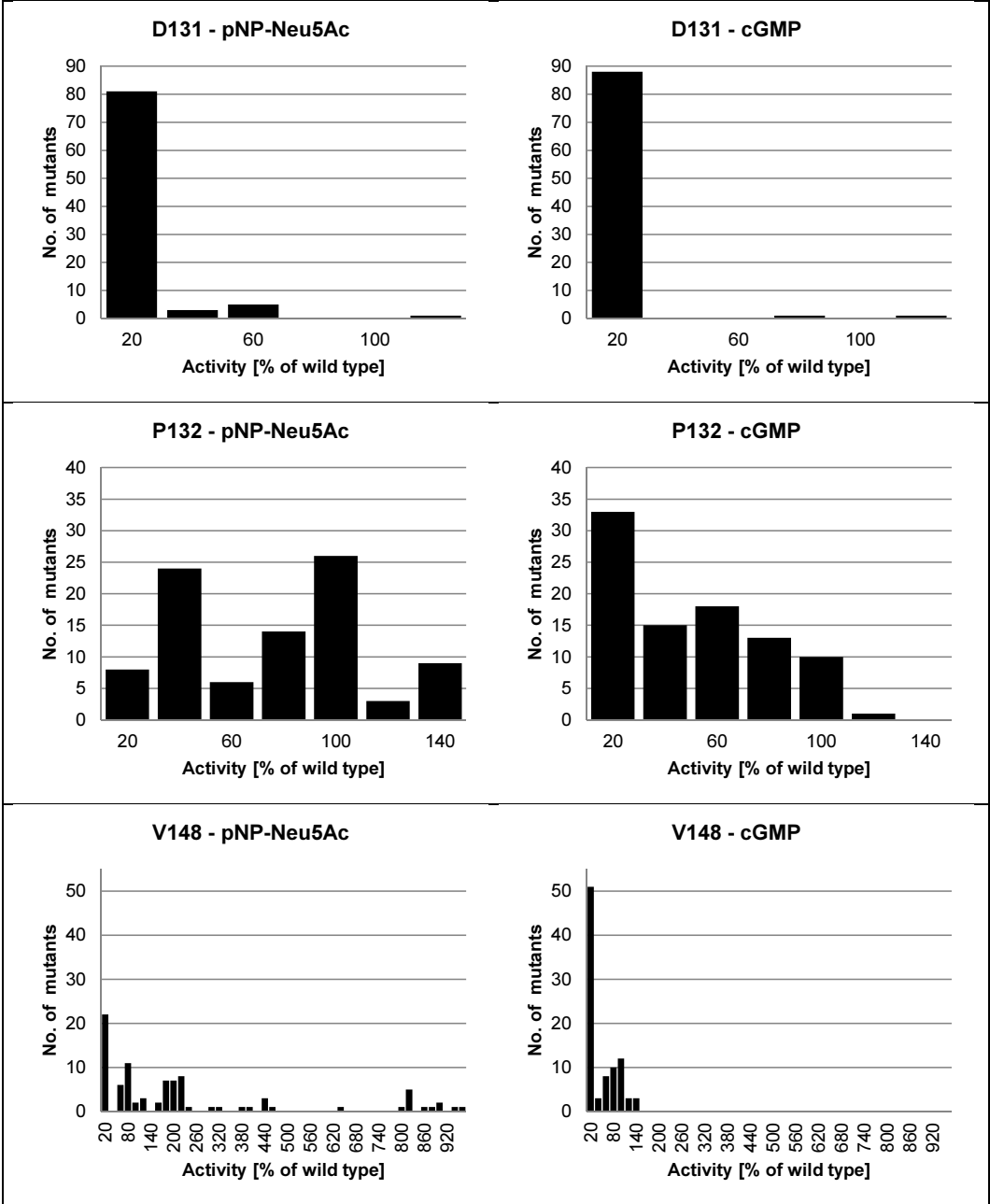
	Mut	n	Activity	
			pNP-NeuAc	cGMP
N66X	Pro	2	218 ± 9	64 ± 2
I69X	Pro	4	351 ± 11	35 ± 12
G90X	Trp	3	280 ± 14	81 ± 11
	Asp	1	265	98
I91X	Pro	3	312 ± 13	16 ± 2
	Tyr	1	296	59
	Glu	1	1422	4
A93X	Thr	4	328 ± 21	87 ± 13
	Ile	1	315	16
S130X	His	3	506 ± 32	4 ± 0
	Ala	2	4301 ± 7	58 ± 1
	Glu	2	292 ± 11	1 ± 0
	Asn	1	277	5
D131X	Glu	2	40 ± 3	0 ± 0
	<i>L126F</i> ¹	1	55	61
P132X	Val	2	134 ± 0	60 ± 15
V148X	His	6	853 ± 58	2 ± 0
	Gln	3	825 ± 36	2 ± 1
	Ile	2	916 ± 27	20 ± 4
	Gly	2	446 ± 9	92 ± 14
Q153X	Ile	2	157 ± 4	116 ± 7
	Asn	1	523	36
	Val	1	159	89
G154X	Asp	8	606 ± 68	36 ± 5
	Glu	2	537 ± 25	32 ± 1
	Pro	1	517	122
	His	1	487	47

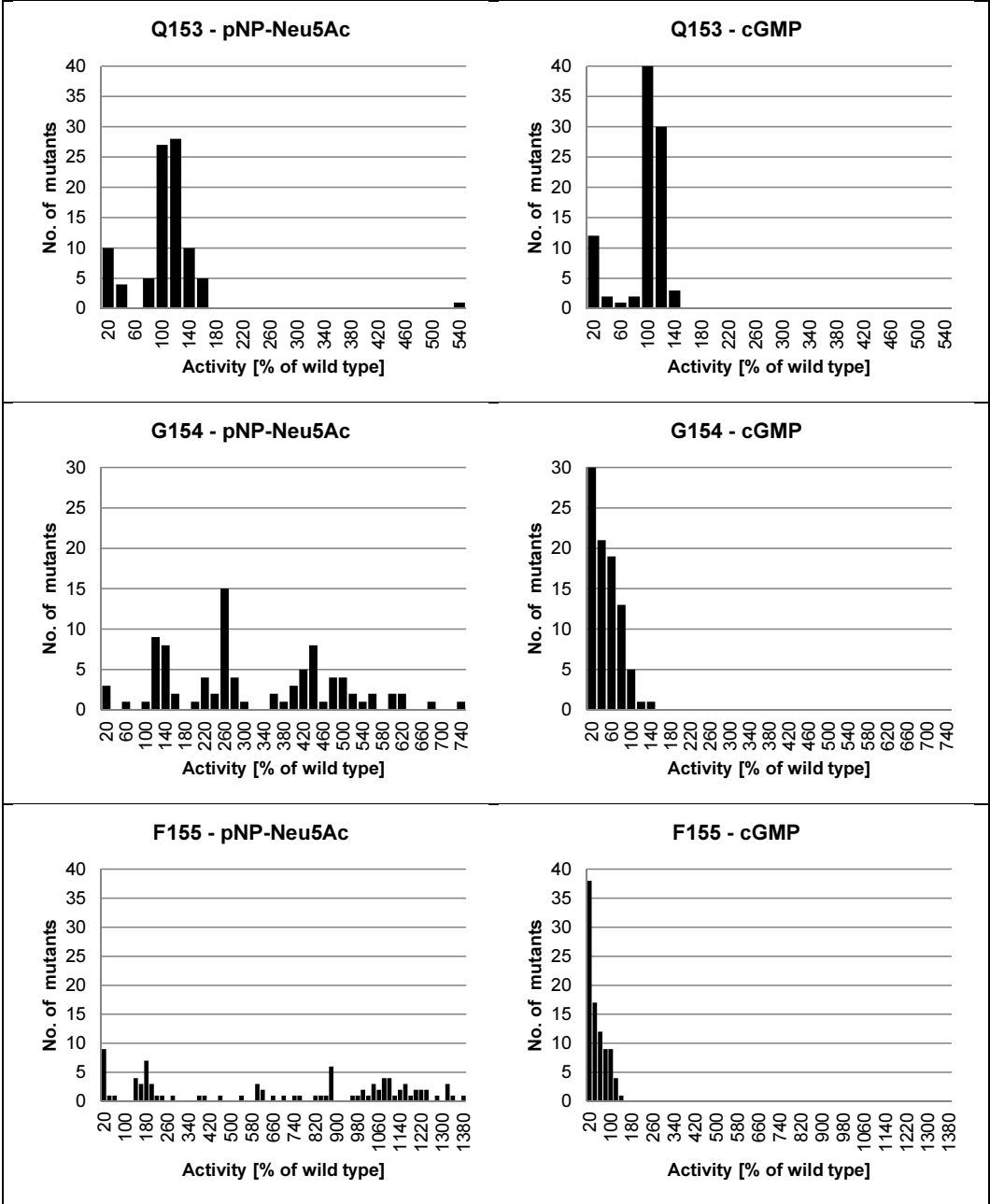
¹ D131X had wild type Asp but unwanted mutation L126F

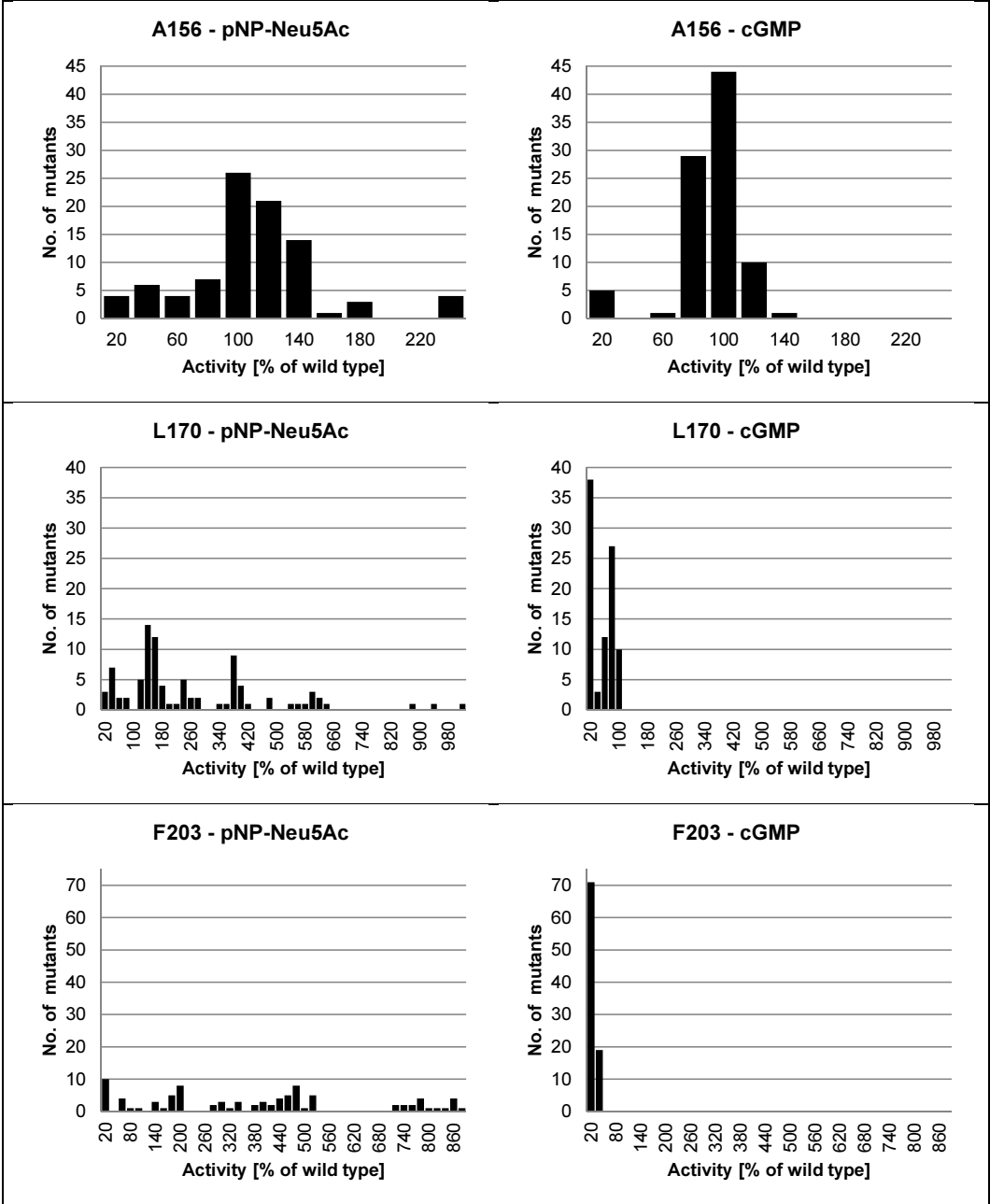
	Mut	n	Activity	
			pNP-NeuAc	cGMP
F155X	Ser	6	1248 ± 60	16 ± 3
	Glu	5	1231 ± 85	29 ± 5
	Ile	3	1181 ± 105	37 ± 7.8
	Thr	3	1159 ± 29	21 ± 12
	Gly	2	1109 ± 36	10 ± 0.5
	Asn	1	1019	41
	Gln	1	1082	30.8
	His	1	683	66
	Val	1	874	69
A156X	Asp	4	222 ± 2	71 ± 6
L170X	His	7	599 ± 20	54 ± 9
	Phe	3	940 ± 69	77 ± 5
	Asp	2	461 ± 1	45 ± 5
	Glu	2	536 ± 14	38 ± 11
F203X	Tyr	4	862 ± 12	28 ± 3
	Ser	2	800 ± 6	8 ± 0
	Gly	2	775 ± 4	11 ± 1.5
	Ala	2	838 ± 16	13 ± 4
	Met	1	515	23
F234X	His	1	505	0.6
	Asp	6	516 ± 26	103 ± 6
	Trp	1	510	31
	Thr	1	402	128
D259X	Glu	7	242 ± 7	0 ± 1
	Val	1	34.8	0
	Ser	1	69	5
N310X	Ile	1	141	74
N311X	Met	2	165 ± 1	37 ± 1
S369X	Asp	4	251 ± 21	83 ± 34
	His	1	256	83
	Arg	1	60	86

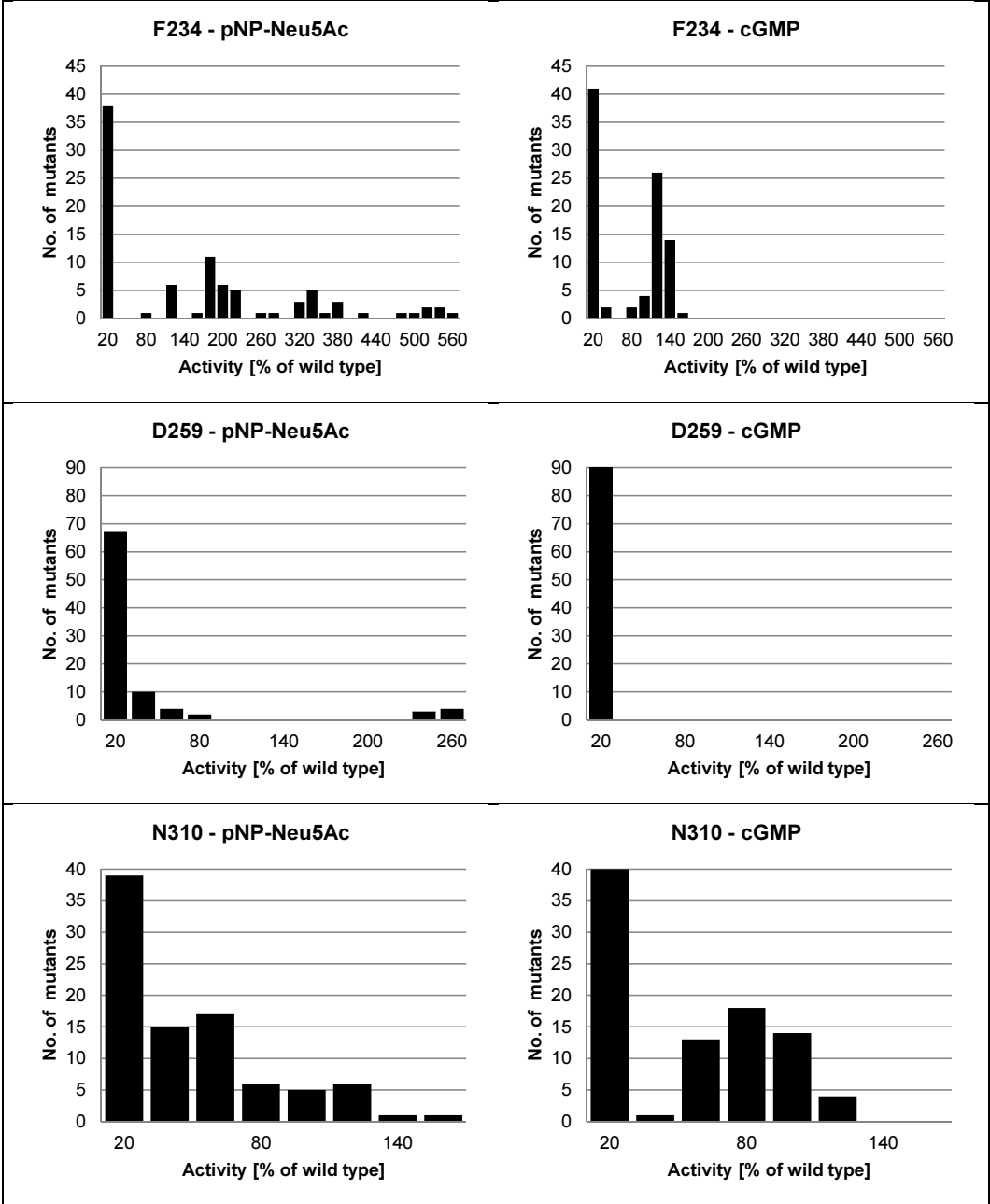












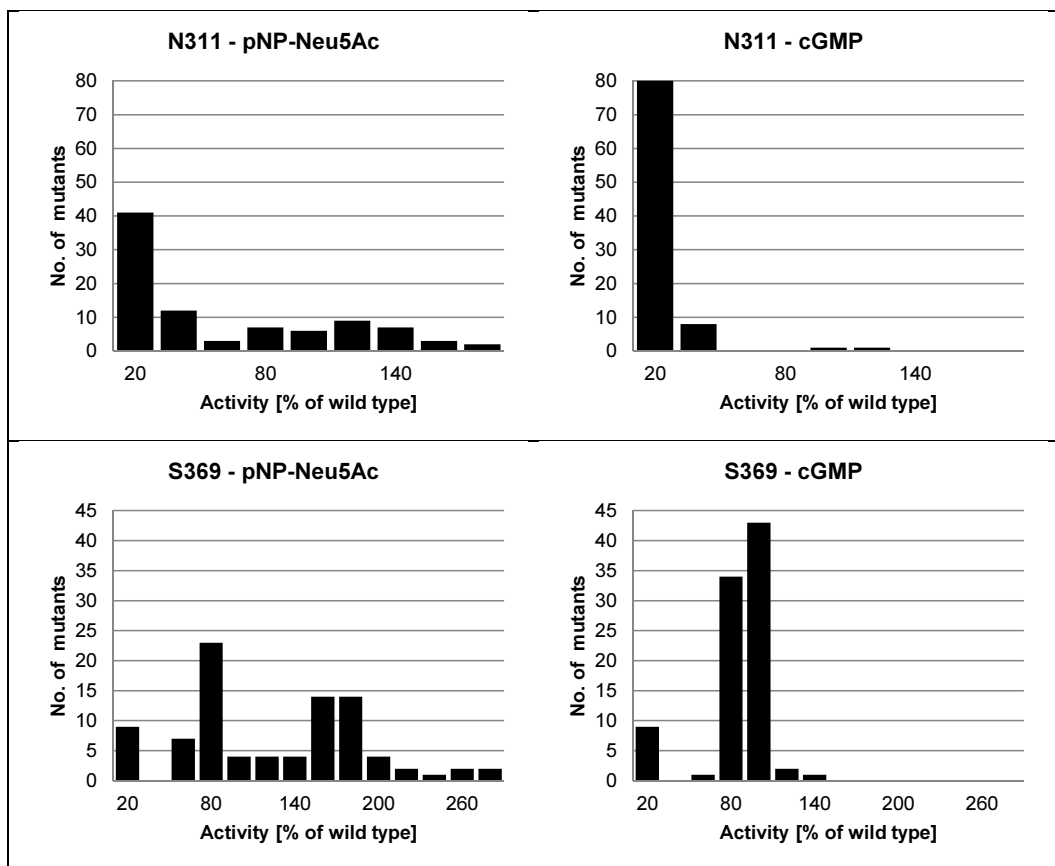


Figure S1. Histogram presenting the activity of screened mutants in individual positions. Activity (% compared to wild type) of 90 mutants tested on pNP-Neu5Ac and cGMP with a bin size of 20 %. 475 and 859 mutants showed no or low activity (0-20 % wild type activity) on pNP-Neu5Ac and cGMP respectively and are not shown in the histograms. Intervals of axes are identical within site (i.e. between pNP-Neu5Ac and cGMP data) but not between sites.

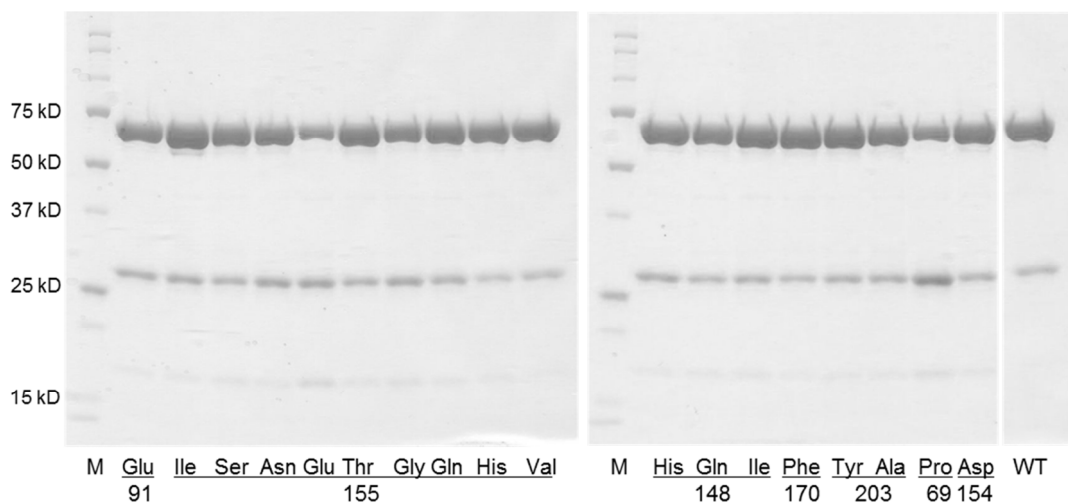


Figure S2. SDS-polyacrylamide gel of 6xHis-tagged mutant enzymes. Enzymes showing increased activity on pNP-Neu5Ac, were purified for further analysis. WT is wild type and 91-Glu is the double mutant I91E/S201L

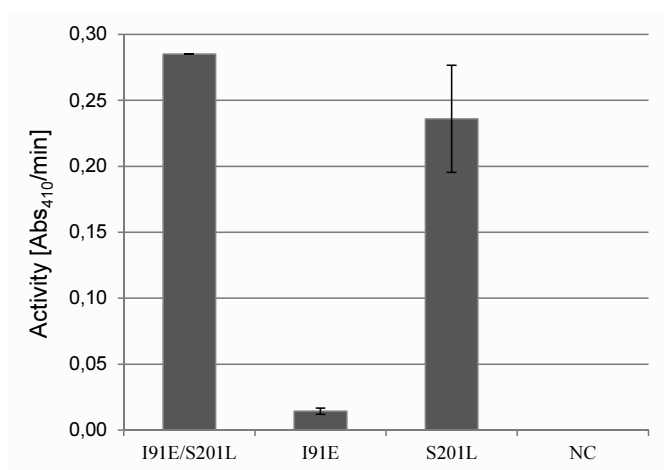


Figure S3. Activity of the double mutant I91E/S201L and the single mutants I91E and S201L on pNP-Neu5Ac. NC is a negative control without enzyme.

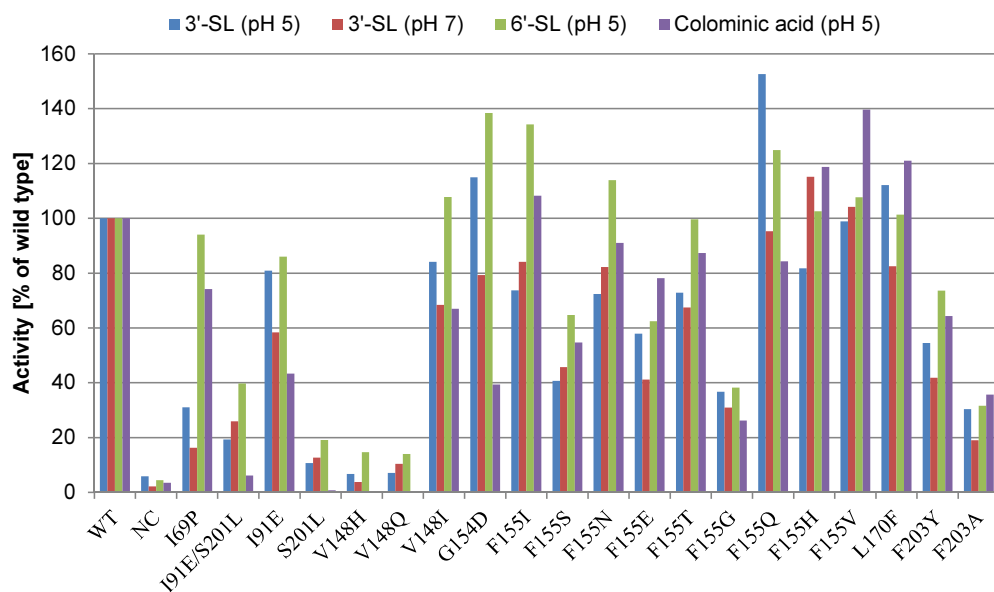


Figure S4. Specific activity of mutants on the natural substrates 3'-sialyllactose (pH 5 and 7), 6'-sialyllactose (pH 5) and colomonic acid (pH 5). Change in activity compared to activity of the wild type sialidase. Negative control (NC) was a reaction without enzyme added. The experiment was repeated twice with same trend, a representative data set is displayed.

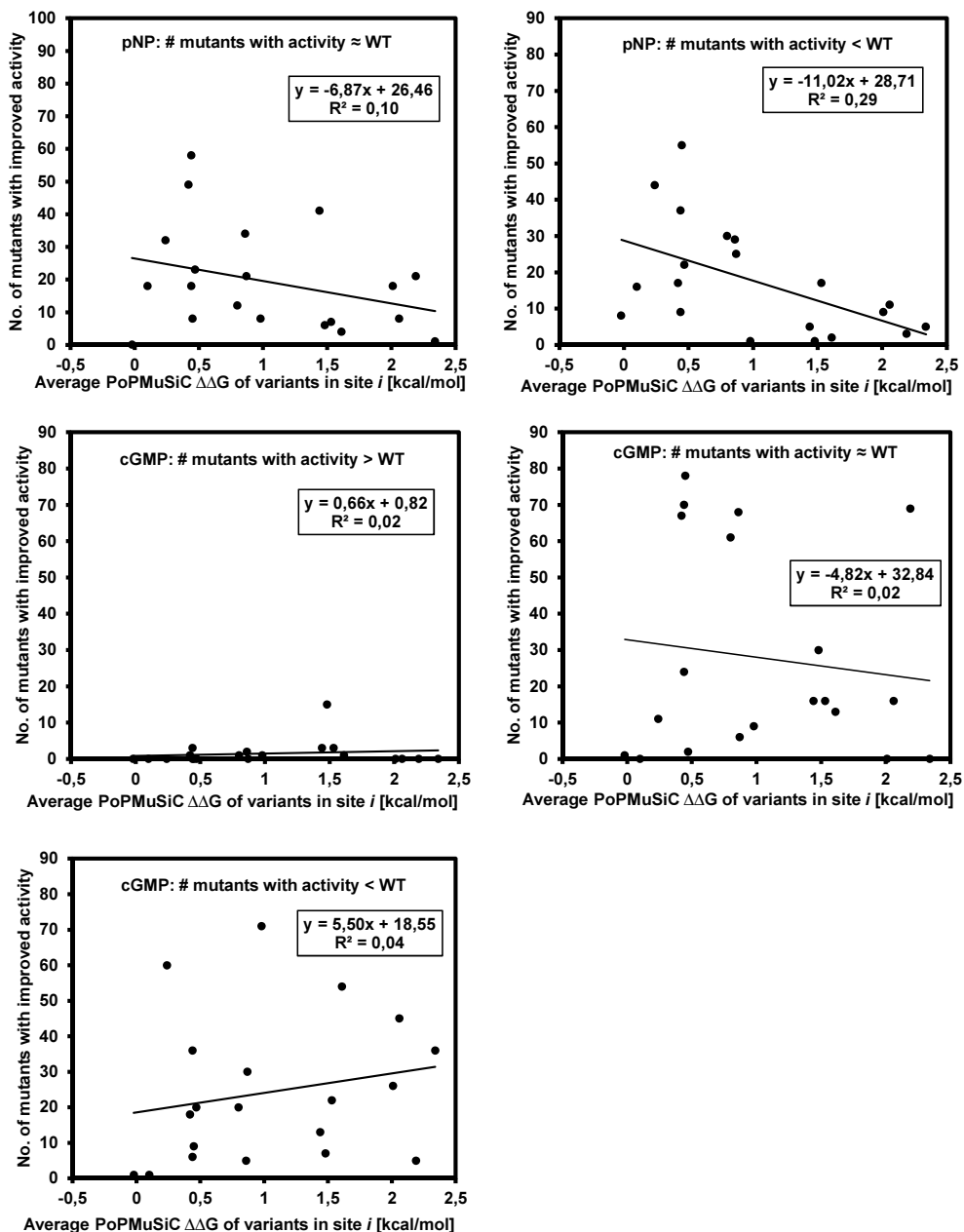


Figure S5. Correlation between PoPMuSiC predictions and activity of mutants. Correlation between potential for chemical variation and observed activity towards hydrolysis of pNP-Neu5Ac or cGMP. PoPMuSiC predictions were used as a measure of potential chemical variation and was for each amino acid position plotted against the number of mutants that exhibited improved activity towards pNP-Neu5Ac.

4. CONCLUDING REMARKS AND PERSPECTIVES

The overall study was aimed to identify biocatalysts (a sialyltransferase and a sialidase), other than the well-known trans-sialidase TcTS, for production of both α -2,3- and α -2,6-sialylated HMOs using a cheap sialyl donor cGMP, as well as to evolve the enzymes for improved catalytic proficiencies (*i.e.*, altered regioselectivity and increased sialidase activity).

Application of trans-sialidase activities of a *P. multocida* sialyltransferase and derived mutant enzymes for production of 3'- and 6'-sialyloligosaccharides

A recombinant *P. multocida* sialyltransferase (PmST) was identified to exhibit α -2,3- and α -2,6-trans-sialidase activities. This is the first study to demonstrate α -2,6-trans-sialidase activity for PmST. Optimum reaction conditions for PmST-catalysed production of 3'-SL and 6'-SL were identified respectively using response surface design. The use of cGMP as donor allowed production of a total SL in a yield of 36%-46%, comparable to that produced in TcTS- (32%) or TrSA₆-catalysed trans-sialylation (47.7%) (Holck et al., 2014; Michalak et al., 2014). However, this was accomplished with the use of a large enzyme concentration (0.35 mg/mL) and a long reaction time (8 h) due to very low trans-sialylation reaction rate of PmST (the $k_{\text{cat}}/K_{\text{m}}$ value for 6'-SL synthesis using 3'-SL as donor was 3134-fold lower than that for α -2,3-SiaT activity using CMP-Neu5Ac as donor).

Based on a study that suggested a specific His residue determines α -2,6-regiospecificity of SiaTs (Kakuta et al., 2008), the mutant PmST_{P34H} was constructed and tested in sialyltransferase and trans-sialylation reactions. PmST_{P34H} had a large increase (980-fold) in α -2,6-SiaT activity compared to the wild type enzyme, while its α -2,3-SiaT activity was almost abolished. Further, with use of cGMP as donor, PmST_{P34H} displayed a distinct preference for 6'-SL synthesis. pH had a great effect on α -2,6-trans-sialidase activity of the wild type enzyme and PmST_{P34H}. In an 8-h reaction using the wild type enzyme, pH 5.4 favored 6'-SL synthesis whereas a much lower activity was observed at pH 6.4. In contrast, for PmST_{P34H}-catalysed 6'-SL synthesis a slightly higher initial reaction rate but also inhibition after 8 h was observed at pH 5.4, and therefore when very long reaction time was used, higher yield was obtainable at pH 6.4. Although the $k_{\text{cat}}/K_{\text{m}}$ value for PmST_{P34H} catalysing 6'-SL synthesis using 3'-SL as donor was similar to that of wild type, the mutant enzyme gave a total SL yield significantly lower than that using the wild type enzyme. This might be due to pH-dependent inactivation effect or the absence of 3'-SL to function as a secondary

donor. The latter would indicate that α -2,3 bound sialic acid in cGMP was not a preferable donor over 3'-SL for α -2,6-trans-sialylation. It will be of interest to compare the α -2,6-trans-sialylation efficacy of the wild type enzyme and PmST_{P34H} with use of other sialyl donors, such as pNP-Neu5Ac, fetuin and colominic acid. We also attempted to produce Sia-GOS using PmST_{P34H} (pH 6.4-40 °C, 20 h) (Fig. S1). However, using NMR failed to determine the distribution between α -2,6 and α -2,3 sialyl linkages due to the structural complexity of GOS and derived Sia-GOS.

Four mutations (M144D, E271F, R313Y and E271F/R313Y) that showed much less sialidase activity but had little effect on SiaT activity for the PmST homologue enzyme (Sugiarto et al., 2011 and 2012) were introduced in PmST. PmST_{M144D} exhibited very low trans-sialidase activity, but maintained α -2,3-SiaT activity. The remaining three mutants catalysed preferentially synthesis of 3'-SL over 6'-SL. Both E271F and R313Y mutations led to reduction in α -2,6-trans-sialidase activity, and combination of the two mutations generated additive effect on reducing this activity. Consequently, PmST_{E271F/R313Y} was the most effective mutant for altering the regioselectivity towards α -2,3-trans-sialylation. This work, together with the study of Choi et al. (2014), indicated the importance of R313 in modulating α -2,3-regioselectivity of PmST.

In this study the trans-sialylation ability of the wild type enzyme and PmST_{P34H} were evaluated for only two acceptors (lactose and GOS). More HMO backbones could be tested as acceptors in the following work. To meet the industrial demand, one would need to investigate the possibility of scaling the process up for production of 3'- and 6'-SLs. As mentioned above, the main drawbacks for application of PmST and derived mutants are the demands of a large concentration of enzyme and a very long reaction time. The former issue can be alleviated by producing the enzyme with use of high-density *E. coli* fermentation, but more importantly efficient immobilization strategies such as membrane immobilization would need to be devised for re-use of the enzyme and enhancing the product yield. Further, protein engineering of PmST for improving the trans-sialidase activities would also be an attractive path.

Site-saturation mutagenesis of *M. viridifaciens* sialidase for improving hydrolase activity

Using semi-rational approach, *M. viridifaciens* sialidase was engineered for improving hydrolase activity. Select active site-amino acids were subjected to site-saturation mutagenesis and mutant libraries were tested on an artificial substrate (pNP-Neu5Ac) and a natural substrate (cGMP). Significant increase in sialidase activity towards pNP-Neu5Ac was acquired whereas for cGMP

only slight improvement was obtained. This is in line with observations that in the case of evolving a promiscuous enzyme, the side activity can be substantially increased with one or few mutations often without significantly affecting the main activity (Khersonsky et al., 2006).

Further, the evidence that none of the single mutants were markedly improved towards natural substrates (cGMP, 3'- and 6'-sialyllactoses and colominc acid) and no correlation was found between activity of mutants on cGMP and the average PoPMuSiC $\Delta\Delta G$ value suggests that this sialidase is likely to have reached proficiency optimum for hydrolysis of galactose-linked sialic acid. In that case, multi-mutants might be required to achieve significant increase in activity towards natural substrates (Tracewell and Arnold, 2009).

In contrast, the observation that the most proficient mutant showed a 27-fold increase in activity on pNP-Neu5Ac and activity of the improved mutants significantly correlated with the predicted $\Delta\Delta G$ value suggested that there is much more room for activity increase towards the artificial substrate and this improvement is associated with sites that possess major chemical changes and decreased stability, consistent with a trade-off between stability and function as widely observed in protein engineering (Tokuriki et al., 2012; Nagatani et al., 2007; Studer et al., 2014).

The differences in enzyme concentrations were not taken into account in the screening because the expression level in microtiter plate was too low to be quantified. In order to verify the screening result on a set of the improved mutants towards pNP-Neu5Ac, the corresponding enzymes were produced in larger-scale, purified and assayed against pNP-Neu5Ac. This largely confirmed the screening results except that the increases in specific activities of these mutants appeared higher than observed in the screen. In the following work, the kinetic analyses could be done for these mutants to investigate whether such improvement is attributed to increased k_{cat} and/or decreased K_{m} . In addition, experimental studies of stability changes for improved mutants could be performed to validate *in silico* predictions.

In conclusion, this work demonstrated for the first time that PmST can catalyse α -2,6-trans-sialylation and that this enzyme (and mutant enzymes) under optimal reaction conditions can catalyse efficient and regioselective synthesis of α -2,3- and α -2,6-sialyloligosaccharides. While a number of questions still need to be resolved, this work has provided a new candidate for larger-scale synthesis of food ingredients such as HMOs. Secondly, this work showed that *M. viridifaciens* sialidase can acquire significant increase in hydrolase activity towards non-natural substrate by

protein engineering and such improvement correlates with increased chemical variation and decreased stability introduced in a given amino acid position. This study has partly explained stability-activity trade-offs as commonly found in protein engineering.

APPENDIX

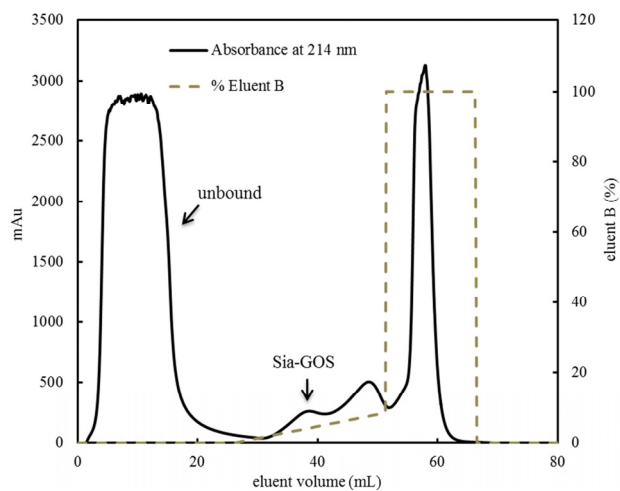


Fig. S1 Purification of Sia-GOS by anion exchange chromatography using a sepharose column. Sia-GOS were produced by PmST_{P34H} catalysis. Eluent B was 1 M sodium chloride.

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